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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US97/00272</p> <p>(22) International Filing Date: 8 January 1997 (08.01.97)</p> <p>(30) Priority Data: 08/584,031 9 January 1996 (09.01.96) US</p> <p>(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).</p> <p>(72) Inventors: ASHKENAZI, Avi, J.; 1456 Tarrytown Street, San Mateo, CA 94402 (US). CHUNTHARAPAI, Anan; 826 Ellis Drive, Colma, CA 94015 (US). KIM, Kyung, Jin; 622 Benvenue Avenue, Los Altos, CA 94024 (US).</p> <p>(74) Agents: MARSCHANG, Diane, L. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: APO-2 LIGAND</p> <p>(57) Abstract</p> <p>A cytokine, designated Apo-2 ligand, which induces mammalian cell apoptosis is provided. The Apo-2 ligand is believed to be a member of the TNF cytokine family. Compositions including Apo-2 ligand chimeras, nucleic acid encoding Apo-2 ligand, and antibodies to Apo-2 ligand are also provided. Methods of using Apo-2 ligand to induce apoptosis and to treat pathological conditions such as cancer, are further provided.</p>		

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Apo-2 LIGANDRELATED APPLICATIONS

This application is a continuation-in-part application of U.S. application serial no. 08/584,031 filed January 9, 1996, the contents of which are incorporated herein by reference.

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FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of a novel cytokine, designated herein as "Apo-2 ligand", which induces mammalian cell apoptosis, to Apo-2 ligand antibodies and to methods of using such compositions.

BACKGROUND OF THE INVENTION

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Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death, however, have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)].

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Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, Science, 267:1445-1449 (1995); Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, *supra*].

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Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, and Apo-1 ligand (also referred to as Fas ligand or CD95 ligand) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995)]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, and Apo-1 ligand have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that

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CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNF-R1) and 75-kDa (TNF-R2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)].

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNF-R1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNF-R1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNF-R1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines. For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

## SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode a novel cytokine, designated "Apo-2 ligand." It is presently believed that Apo-2 ligand is a member of the TNF cytokine family; Apo-2 ligand is related in amino acid sequence to some known TNF-related proteins, including the Apo-1 ligand. Applicants

found, however, that the Apo-2 ligand is not inhibited appreciably by known soluble Apo-1 or TNF receptors, such as the Fas/Apo-1, TNF-R1, or TNF-R2 receptors.

In one embodiment, the invention provides isolated biologically active Apo-2 ligand. In particular, the invention provides isolated biologically active Apo-2 ligand which includes an amino acid sequence comprising residues 114-281 of Figure 1A. In another embodiment, the Apo-2 ligand includes an amino acid sequence comprising residues 41-281 or 15-281 of Figure 1A. In another embodiment, the isolated biologically active Apo-2 ligand includes an amino acid sequence shown as residues 1-281 of Figure 1A (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 ligand fused to another, heterologous polypeptide. An example of such a chimeric molecule comprises the Apo-2 ligand fused to a tag polypeptide sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 ligand. In one aspect, the nucleic acid molecule is RNA or DNA that encodes a biologically active Apo-2 ligand or is complementary to a nucleic acid sequence encoding such Apo-2 ligand, and remains stably bound to it under at least moderately stringent conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1A that codes for the full-length protein from residue 1 to residue 281 (i.e., nucleotides 91 through 933), inclusive, or nucleotides 211 through 933 that encodes for the extracellular protein from residue 41 to 281, inclusive, or nucleotides 430 through 933 that encodes for the extracellular protein from residue 114 to 281, inclusive, of the nucleic acid sequence shown in Figure 1A (SEQ ID NO:2); or

(b) a sequence corresponding to the sequence of (a) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a replicable vector comprising the nucleic acid molecule encoding the Apo-2 ligand operably linked to control sequences recognized by a host cell transfected or transformed with the vector. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 ligand which comprises culturing a host cell comprising the nucleic acid molecule and recovering the protein from the host cell culture is further provided.

In another embodiment, the invention provides an antibody which binds to the Apo-2 ligand. In one aspect, the antibody is a monoclonal antibody having antigen specificity for Apo-2 ligand.

In another embodiment, the invention provides a composition comprising biologically active Apo-2 ligand and a pharmaceutically-acceptable carrier. The composition may be a pharmaceutical composition useful for inducing or stimulating apoptosis.

In another embodiment, the invention provides a method for inducing apoptosis in mammalian cells, comprising exposing mammalian cells, *in vivo* or *ex vivo*, to an amount of Apo-2 ligand effective for inducing apoptosis.

In another embodiment, the invention provides methods of treating a mammal having cancer. In the methods, an effective amount of Apo-2 ligand is administered to a mammal diagnosed as having cancer.

The Apo-2 ligand may also be administered to the mammal along with one or more other therapies, such as chemotherapy, radiation therapy, or other agents capable of exerting anti-tumor activity.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 ligand or Apo-2 ligand antibodies. The articles of manufacture and kits include a container, a label on the container, and a composition contained within the container. The label on the container indicates that the composition can be used for certain therapeutic or non-therapeutic applications. The composition contains an active agent, and the active agent comprises Apo-2 ligand or Apo-2 ligand antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the nucleotide sequence of human Apo-2 ligand cDNA and its derived amino acid sequence.

Figure 1B shows an alignment of the C-terminal region of human Apo-2 ligand with the corresponding region of known members of the human TNF cytokine family, 4-1BBL, OX40L, CD27L, CD30L, TNF- $\alpha$ , LT- $\beta$ , LT- $\alpha$ , CD40L, and Apo-1L.

Figures 1C-1E show (C) the cellular topology of the recombinant, full-length, C-terminal myc epitope-tagged Apo-2 ligand expressed in human 293 cells, as determined by FACS analysis using anti-myc epitope antibody; (D) the size and subunit structure of recombinant, His<sub>10</sub> epitope-tagged soluble Apo-2 expressed in recombinant baculovirus-infected insect cells and purified by Ni<sup>2+</sup>-chelate affinity chromatography, as determined with (lanes 2, 3) or without (lane 1) chemical crosslinking followed by SDS-PAGE and silver staining; (E) the size and subunit structure of recombinant, gD epitope-tagged, soluble Apo-2 ligand expressed in metabolically labeled human 293 cells, as determined by immunoprecipitation with anti-gD epitope antibody, followed by SDS-PAGE and autoradiography.

Figures 2A-2E show the induction of apoptosis in B and T lymphocyte cell lines by Apo-2 ligand. Apoptotic cells were identified by characteristic morphological changes (A); by positive fluorescence staining with propidium iodide (PI) and FITC-conjugated annexin V, measured by flow cytometry (B-D); and by analysis of internucleosomal DNA fragmentation (E).

Figures 3A-3C show the time course and the dose-dependence of Apo-2 ligand-induced apoptosis and the lack of inhibition of Apo-2 ligand-induced apoptosis by soluble receptor-IgG-fusion proteins based on the Fas/Apo-1 receptor, TNF-R1 receptor, or TNF-R2 receptor.

Figure 4 shows the expression of Apo-2 ligand mRNA in human fetal and human adult tissues, as measured by Northern blot analysis.

Figure 5 shows the *in vivo* effect of Apo-2 ligand, administered by intratumor injection, alone or in combination with Doxorubicin, on the weight of human MDA231 breast carcinoma-based tumors grown in nude mice.

Figure 6 shows the *in vivo* effect of Apo-2 ligand, administered by intratumor injection, alone or in combination with 5-FU, on the weight of human HCT116 colon carcinoma-based tumors grown in nude mice.

Figure 7 shows the *in vivo* effect of Apo-2 ligand, administered by intraperitoneal injection, alone or in combination with 5-FU, on the size of human HCT116 colon carcinoma-based tumors grown in nude mice.

Figure 8 shows the *in vivo* effect of Apo-2 ligand, administered by intraperitoneal injection, alone or in combination with 5-FU, on the weight of human HCT116 colon carcinoma-based tumors grown in nude mice.

Figure 9 is a bar diagram illustrating that CrmA but not dominant negative FADD blocks Apo-2 ligand-induced apoptosis in HeLa-S3 cells.

Figure 10 shows FACS analysis of apoptosis induced by Apo-2 ligand and the effect of four anti-Apo-2 ligand antibodies: 1D1, 2G6, 2E11, and 5C2 (apoptotic 9D cells detected using FITC-conjugated annexin V - bold line; live, unstained cells - thin line).

Figure 11 is a bar diagram illustrating antigen specificity of monoclonal antibodies 1D1, 2G6, 2E11, and 5C2.

Figure 12 is a bar diagram illustrating the results of an epitope mapping assay of monoclonal antibodies 1D1, 2G6, 2E11, and 5C2.

Figure 13 is a bar diagram illustrating the results of an assay testing the ability of monoclonal antibody 1D1 to bind to several different synthetic peptides consisting of specific amino acid regions of the Apo-2 ligand.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### 1. Definitions

The terms "Apo-2 ligand" and "Apo-2L" are used herein to refer to a polypeptide sequence which includes amino acid residues 114-281, inclusive, residues 41-281, inclusive, residues 15-281, inclusive, or residues 1-281, inclusive, of the amino acid sequence shown in Figure 1A, as well as biologically active deletional, insertional, or substitutional variants of the above sequences. In a preferred embodiment, the polypeptide sequence has at least residues 114-281 of Figure 1A. In another preferred embodiment, the biologically active variants have at least about 80% sequence identity, more preferably at least about 90% sequence identity, and even more preferably, at least about 95% sequence identity with any one of the above sequences. The definition encompasses Apo-2 ligand isolated from an Apo-2 ligand source, such as from the human tissue types described herein (see Example 8) or from another source, or prepared by recombinant or synthetic methods. The present definition of Apo-2 ligand excludes known EST sequences, such as GenBank HHEA47M, T90422, R31020, H43566, H44565, H44567, H54628, H44772, H54629, T82085, and T10524.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2 ligand, or a portion thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2 ligand. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous



solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein *in situ* within recombinant cells, since at least one component of the Apo-2 ligand natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

An "isolated" Apo-2 ligand nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 ligand nucleic acid. An isolated Apo-2 ligand nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 ligand nucleic acid molecules therefore are distinguished from the Apo-2 ligand nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 ligand nucleic acid molecule includes Apo-2 ligand nucleic acid molecules contained in cells that ordinarily express Apo-2 ligand where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 ligand monoclonal antibodies (including agonist and antagonist antibodies) and anti-Apo-2 ligand antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 ligand antibody with a constant domain

(e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

"Biologically active" for the purposes herein to characterize Apo-2 ligand means having the ability to induce or stimulate apoptosis in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, leukemia, blastoma, and sarcoma. More particular examples of such cancers include squamous cell carcinoma, small-cell lung cancer, non-small cell lung cancer, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, stomach cancer, bladder cancer, hepatoma, breast cancer,

colon carcinoma, and head and neck cancer. In one embodiment, the cancer includes follicular lymphoma, carcinoma with *p53* mutations, or hormone-dependent cancer such as breast cancer, prostate cancer, or ovarian cancer.

5 The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## II. Compositions and Methods of the Invention

10 The present invention provides a novel cytokine related to the TNF ligand family, the cytokine identified herein as "Apo-2 ligand." The predicted mature amino acid sequence of human Apo-2 ligand contains 281 amino acids, and has a calculated molecular weight of approximately 32.5 kDa and an isoelectric point of approximately 7.63. There is no apparent signal sequence at the N-terminus, although hydropathy analysis indicates the presence of a hydrophobic region between residues 15 and 40. The absence of a signal sequence and the presence of an internal hydrophobic region suggests that Apo-2 ligand is a type  
15 II transmembrane protein. A potential N-linked glycosylation site is located at residue 109 in the putative extracellular region. The putative cytoplasmic region comprises amino acid residues 1-14, the transmembrane region comprises amino acid residues 15-40 and the extracellular region comprises amino acid residues 41-281, shown in Figure 1A. An Apo-2 ligand polypeptide comprising amino acid residues 114-281 of the extracellular region, shown in Figure 1A, is also described in the Examples below.

### 20 A. Preparation of Apo-2 Ligand

The description below relates primarily to production of Apo-2 ligand by culturing cells transformed or transfected with a vector containing Apo-2 ligand nucleic acid and recovering the polypeptide from the cell culture. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2 ligand.

#### 25 1. Isolation of DNA Encoding Apo-2 Ligand

The DNA encoding Apo-2 ligand may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 ligand mRNA and to express it at a detectable level. Accordingly, human Apo-2 ligand DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage library of human placental cDNA described in Example 1. The Apo-2 ligand-encoding gene  
30 may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 ligand or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Examples of oligonucleotide probes are provided in Example 1. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al.,  
35 Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 ligand is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library with

two different oligonucleotide probes. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous so that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP, biotinylation or enzyme labeling.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Amino acid sequence variants of Apo-2 ligand can be prepared by introducing appropriate nucleotide changes into the Apo-2 ligand DNA, or by synthesis of the desired Apo-2 ligand polypeptide. Such variants represent insertions, substitutions, and/or deletions of residues within or at one or both of the ends of the intracellular region, the transmembrane region, or the extracellular region, or of the amino acid sequence shown for the full-length Apo-2 ligand in Figure 1A. Any combination of insertion, substitution, and/or deletion can be made to arrive at the final construct, provided that the final construct possesses the desired apoptotic activity as defined herein. In a preferred embodiment, the variants have at least about 80% sequence identity, more preferably, at least about 90% sequence identity, and even more preferably, at least about 95% sequence identity with the sequences identified herein for the intracellular, transmembrane, or extracellular regions of Apo-2 ligand, or the full-length sequence for Apo-2 ligand. The amino acid changes also may alter post-translational processes of the Apo-2 ligand, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the Apo-2 ligand sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding native or variant Apo-2 ligand may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

### (i) Signal Sequence Component

The Apo-2 ligand may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 ligand DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including

*Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 ligand presequence that normally directs insertion of Apo-2 ligand in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2 ligand.

10

#### (ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

20

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 ligand DNA. However, the recovery of genomic DNA encoding Apo-2 ligand is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 ligand DNA.

25

#### (iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and

thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)) or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin  
5 (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 ligand nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing  
10 the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2 ligand. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 ligand are synthesized from the amplified DNA. Other examples of amplifiable  
15 genes include metallothionein-I and -II, adenosine deaminase, and ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA,  
20 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2 ligand. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

25 Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2 ligand, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

30 A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting  
35 transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den

Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

5 Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 ligand nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the Apo-2 ligand nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes,  
10 inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 ligand encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated  
15 promoter sequence into the vector. Both the native Apo-2 ligand promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 ligand DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776],  
20 and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 ligand [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding  
25 Apo-2 ligand.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that  
30 may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase,  
35 glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2,

isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

5 Apo-2 ligand transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock  
10 promoters, and from the promoter normally associated with the Apo-2 ligand sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402  
15 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-  
20 601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon  $\beta$  1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous  
25 sarcoma virus long terminal repeat as a promoter].

#### (v) Enhancer Element Component

Transcription of a DNA encoding Apo-2 ligand by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and  
30 position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the  
35 SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 ligand-encoding sequence, but is preferably located at a site 5' from the promoter.



(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2 ligand.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 ligand may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of Apo-2 ligand that are biologically active Apo-2 ligand.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 ligand in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of Apo-2 ligand is pRK5 [EP 307,247; also described in Example 1] or pSVI6B [WO 91/08291 published 13 June 1991].

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas*

such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2 ligand-encoding vectors. *Saccharomyces cerevisiae*, or common  
5 baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 ligand are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture.  
10 Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594  
15 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* ("Sf9") cells, described in Example 2.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be  
20 utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the Apo-2 ligand-encoding DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 ligand is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2 ligand-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are  
25 available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See,  
30 e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather,  
35 Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 ligand production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

5 Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable,  
10 either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859  
15 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are  
20 typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 25 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 ligand may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 ligand may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium  
30 ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar  
35 range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

5 The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an  
10 appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorsceners or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes,  
15 including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate  
20 directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be  
25 either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native Apo-2 ligand polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 ligand DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Ligand Polypeptide

30 Apo-2 ligand preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the Apo-2 ligand is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When Apo-2 ligand is produced in a recombinant cell other than one of human origin, the  
35 Apo-2 ligand is free of proteins or polypeptides of human origin. However, it is usually necessary to purify Apo-2 ligand from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2 ligand. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 ligand thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-

exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

In a preferred embodiment, the Apo-2 ligand can be isolated by affinity chromatography, as  
5 described in Example 3.

Apo-2 ligand variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native Apo-2 ligand, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of an Apo-2 ligand fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing  
10 antibody to the antigen can be used to adsorb the fusion polypeptide. In a preferred embodiment, an extracellular sequence of Apo-2 ligand is fused to a His<sub>10</sub> peptide and purified by Ni<sup>2+</sup>-chelate affinity chromatography.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of  
15 adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native Apo-2 ligand may require modification to account for changes in the character of Apo-2 ligand or its variants upon expression in recombinant cell culture.

#### 7. Covalent Modifications of Apo-2 Ligand Polypeptides

Covalent modifications of Apo-2 ligand are included within the scope of this invention. Both  
20 native Apo-2 ligand and amino acid sequence variants of the Apo-2 ligand may be covalently modified. One type of covalent modification of the Apo-2 ligand is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 ligand with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2 ligand.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 ligand to a water-  
25 insoluble support matrix or surface for use in the method for purifying anti-Apo-2 ligand antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide  
30 date yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutamyl and asparaginy residues to the  
35 corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 ligand polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native Apo-2 ligand, and/or adding one or more glycosylation sites that are not present in the native Apo-2 ligand.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 ligand polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native Apo-2 ligand sequence (for O-linked glycosylation sites). The Apo-2 ligand amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 ligand polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, *supra*.

Another means of increasing the number of carbohydrate moieties on the Apo-2 ligand polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 ligand polypeptide may be accomplished chemically or enzymatically. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 ligand comprises linking the Apo-2 ligand polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth, for instance, in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 5                   8.       Epitope-tagged Apo-2 Ligand

The present invention also provides chimeric polypeptides comprising Apo-2 ligand fused to another, heterologous polypeptide. In one embodiment, the chimeric polypeptide comprises a fusion of the Apo-2 ligand with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2 ligand. The  
10       presence of such epitope-tagged forms of the Apo-2 ligand can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2 ligand to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)];  
15       the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the  
20       T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 ligand may be constructed and produced according to the methods described above for native and variant Apo-2 ligand. Apo-2 ligand-tag polypeptide fusions are  
25       preferably constructed by fusing the cDNA sequence encoding the Apo-2 ligand portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2 ligand-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 ligand will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. Examples of epitope-tagged Apo-2 ligand are described in  
30       further detail in Example 2 below.

Epitope-tagged Apo-2 ligand can be purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene). The epitope-tagged Apo-2 ligand can then be eluted from the affinity column using techniques known in the art.

#### 35                   B.       Therapeutic Uses for Apo-2 Ligand

Apo-2 ligand, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. Generally, the methods for inducing apoptosis in mammalian cells comprise exposing the cells to an effective amount of Apo-2 ligand. This can be accomplished *in vivo* or *ex vivo* in accordance, for instance, with the methods described below and in the Examples. It is contemplated

that the methods for inducing apoptosis can be employed in therapies for particular pathological conditions which are characterized by decreased levels of apoptosis. Examples of such pathological conditions include autoimmune disorders like lupus and immune-mediated glomerular nephritis, and cancer. Therapeutic application of Apo-2 ligand for the treatment of cancer is described in detail below.

5 In the methods for treating cancer, Apo-2 ligand is administered to a mammal diagnosed as having cancer. It is of course contemplated that the Apo-2 ligand can be employed in combination with still other therapeutic compositions and techniques, including other apoptosis-inducing agents, chemotherapy, radiation therapy, and surgery.

The Apo-2 ligand is preferably administered to the mammal in a pharmaceutically-acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th  
10 ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7.4 to about 7.8. It will be apparent to those persons  
15 skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of Apo-2 ligand being administered.

The Apo-2 ligand can be administered to the mammal by injection (*e.g.*, intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. It is also contemplated that the Apo-2 ligand can be administered by *in*  
20 *vivo* or *ex vivo* gene therapy.

Effective dosages and schedules for administering Apo-2 ligand may be determined empirically, and making such determinations is within the skill in the art. It is presently believed that an effective dosage or amount of Apo-2 ligand used alone may range from about 1  $\mu\text{g/kg}$  to about 100  $\text{mg/kg}$  of body weight or more per day. Interspecies scaling of dosages can be performed in a manner known in the art,  
25 *e.g.*, as disclosed in Mordenti et al., Pharmaceut. Res., 8:1351 (1991). Those skilled in the art will understand that the dosage of Apo-2 ligand that must be administered will vary depending on, for example, the mammal which will receive the Apo-2 ligand, the route of administration, and other drugs or therapies being administered to the mammal.

The one or more other therapies administered to the mammal may include but are not limited  
30 to, chemotherapy and/or radiation therapy, immunoadjuvants, cytokines, and antibody-based therapies. Examples include interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-6), leukemia inhibitory factor, interferons, TGF-beta, erythropoietin, thrombopoietin, anti-VEGF antibody and HER-2 antibody. Other agents known to induce apoptosis in mammalian cells may also be employed, and such agents include TNF- $\alpha$ , TNF- $\beta$  (lymphotoxin- $\alpha$ ), CD30 ligand, 4-1BB ligand, and Apo-1 ligand.

35 Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil ("5-FU"), etoposide, camptothecin, Leucovorin, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepe, Busulfan, Cytosin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. Preparation and dosing schedules for such chemotherapy may be used according to manufacturers' instructions or as determined empirically by



the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992).

The chemotherapy is preferably administered in a pharmaceutically-acceptable carrier, such as those described above for Apo-2 ligand. The mode of administration of the chemotherapy may be the same as employed for the Apo-2 ligand or it may be administered to the mammal via a different mode. For example, the Apo-2 ligand may be injected while the chemotherapy is administered orally to the mammal. Modes of administering chemotherapy in combination with Apo-2 ligand are described in further detail in Examples 9-12 below.

Radiation therapy can be administered to the mammal according to protocols commonly employed in the art and known to the skilled artisan. Such therapy may include cesium, iridium, iodine, or cobalt radiation. The radiation therapy may be whole body irradiation, or may be directed locally to a specific site or tissue in or on the body. Typically, radiation therapy is administered in pulses over a period of time from about 1 to about 2 weeks. The radiation therapy may, however, be administered over longer periods of time. Optionally, the radiation therapy may be administered as a single dose or as multiple, sequential doses.

The Apo-2 ligand and one or more other therapies may be administered to the mammal concurrently or sequentially. Following administration of Apo-2 ligand and one or more other therapies to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically, by biopsy or by standard x-ray imaging techniques.

It is contemplated that Apo-2 ligand can be employed to treat cancer cells *ex vivo*. Such *ex vivo* treatment may be useful in bone marrow transplantation and particularly, autologous bone marrow transplantation. For instance, treatment of cells or tissue(s) containing cancer cells with Apo-2 ligand, and optionally, with one or more other therapies, such as described above, can be employed to induce apoptosis and substantially deplete the cancer cells prior to transplantation in a recipient mammal.

Cells or tissue(s) containing cancer cells are first obtained from a donor mammal. The cells or tissue(s) may be obtained surgically and preferably, are obtained aseptically. In the method of treating bone marrow for transplantation, bone marrow is obtained from the mammal by needle aspiration. The cells or tissue(s) containing cancer cells are then treated with Apo-2 ligand, and optionally, with one or more other therapies, such as described above. Bone marrow is preferably fractionated to obtain a mononuclear cell fraction (such as by centrifugation over ficoll-hypaque gradient) prior to treatment with Apo-2 ligand.

The treated cells or tissue(s) can then be infused or transplanted into a recipient mammal. The recipient mammal may be the same individual as the donor mammal or may be another, heterologous mammal. For an autologous bone marrow transplant, the mammal is treated prior to the transplant with an effective dose of radiation or chemotherapy as known in the art and described for example in Autologous Bone Marrow Transplantation: Proceedings of the Third International Symposium, Dicke et al., eds., University of Texas M.D. Anderson Hospital and Tumor Institute (1987).

C. Non-Therapeutic Uses for Apo-2 Ligand

The Apo-2 ligand of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 ligand may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 ligand is present in the cell type(s) being evaluated. Apo-2 ligand nucleic acid will also be useful for the preparation of Apo-2 polypeptide by the recombinant techniques described herein.

The isolated Apo-2 ligand may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 ligand may be prepared. Apo-2 ligand preparations are also useful in generating antibodies, as standards in assays for Apo-2 ligand (e.g., by labeling Apo-2 ligand for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques for example, in identifying or in isolating a receptor that binds Apo-2 ligand, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Nucleic acids which encode Apo-2 ligand can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 ligand or an appropriate sequence thereof can be used to clone genomic DNA encoding Apo-2 ligand in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2 ligand. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 ligand transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 ligand introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2 ligand.

Alternatively, non-human homologues of Apo-2 ligand can be used to construct a Apo-2 ligand "knock out" animal which has a defective or altered gene encoding Apo-2 ligand as a result of homologous recombination between the endogenous gene encoding Apo-2 ligand and altered genomic DNA encoding Apo-2 ligand introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 ligand can be used to clone genomic DNA encoding Apo-2 ligand in accordance with established techniques. A portion of the genomic DNA encoding Apo-2 ligand can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, *Cell*, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., *Cell*, 69:915 (1992)].

The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 ligand polypeptide.

#### D. Anti-Apo-2 Ligand Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 ligand may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

##### 1. Polyclonal Antibodies

The Apo-2 ligand antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 ligand polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

##### 2. Monoclonal Antibodies

The Apo-2 ligand antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 ligand polypeptide or a fusion protein thereof. Cells expressing Apo-2 ligand at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103]. Immortalized cell lines

are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications; Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2 ligand. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA), fluorescein activated cell sorting (FACS) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art, and are described further in the Examples below. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Rodbard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In one embodiment of the invention, the monoclonal antibodies may include the 1D1, 2G6, 2E11, or 5C2 antibodies described herein and in the Examples below. The monoclonal antibodies may also include antibodies having the same biological characteristics as the 1D1, 2G6, 2E11, or 5C2 monoclonal antibodies secreted by the hybridoma cell lines deposited under American Type Culture Collection Accession Nos. ATCC \_\_\_\_\_, \_\_\_\_\_, \_\_\_\_\_, or \_\_\_\_\_, respectively. The term "biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities of the monoclonal antibody, e.g., ability to substantially reduce or inhibit Apo-2 ligand-induced apoptosis or substantially reduce or block binding of Apo-2 ligand to its receptor. The antibody preferably binds to the same epitope as, or to substantially the same epitope as, the

1D1, 2G6, 2E11, or 5C2 antibodies disclosed herein. This can be determined by conducting assays described herein and in the Examples.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., *supra*] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

*In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain ( $CH_1$ ) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain  $CH_1$  domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 ligand antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab',  $F(ab')_2$  or other antigen-binding

subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)] and the method of Queen et al., Proc. Natl. Acad. Sci., 86:10029-10033 (1989) using computer modeling, by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art.

Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR  
5 residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full  
10 repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immunol., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol.,  
15 147(1):86-95 (1991)).

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2 ligand, the other one is for any other antigen, and preferably for a cell-surface protein or receptor  
25 or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas  
30 (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the  
35 desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate

expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### E. Uses of Apo-2 Ligand Antibodies

Apo-2 ligand antibodies may be used in diagnostic assays for Apo-2 ligand, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 ligand antibodies also are useful for the affinity purification of Apo-2 ligand from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 ligand are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 ligand to be purified, and



thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2 ligand, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 ligand from the antibody. Apo-2 ligand antibodies also are useful for the affinity purification of a solubilized Apo-2 receptor or for expression cloning of an Apo-2 receptor.

The antibodies disclosed herein may also be employed as therapeutics. For instance, anti-Apo-2 ligand antibodies which block Apo-2 ligand activity (like Apo-2 ligand-induced apoptosis) may be employed to treat pathological conditions or diseases associated with increased apoptosis [see, Thompson, *supra*].

10 F. Kits Containing Apo-2 Ligand or Apo-2 Ligand Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 ligand or Apo-2 ligand antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 ligand or an Apo-2 ligand antibody. The label on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

20 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

\*\*\*\*\*

25 The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All references cited in the present specification are hereby incorporated by reference in their entirety.

**EXAMPLES**

30 All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

**EXAMPLE 1**

35 Isolation of cDNA Clones Encoding Human Apo-2 Ligand

To isolate a full-length cDNA for Apo-2 ligand, a lambda gt11 bacteriophage library of human placental cDNA (about  $1 \times 10^6$  clones) (HL10756, commercially available from Clontech) was screened by hybridization with synthetic oligonucleotide probes based on an EST sequence (GenBank locus HHEA47M), which showed some degree of homology to human Fas/Apo-1 ligand. The EST sequence of

HHEA47M is 390 bp and when translated in its +3 frame, shows 16 identities to a 34 amino acid region of human Apo-1 ligand. The sequence of HHEA47M is as follows:

GGGACCCCAATGACGAAGAGAGTATGAACAGCCCCTGCTGGCAAGTCAAGTGGCAACTCCGT  
CAGCTCGTTAGAAAGATGATTTTGAGAACCTCTGAGGAAACCATTTCTACAGTTCAAGAAAAG  
5 CAACAAAATATTTCTCCCCTAGTGAGAGAAAGAGGTCCTCAGAGAGTAGCAGCTCACATAACT  
GGGACCAGAGGAAGAAGCAACACATTGTCTTCTCCAAACTCCAAGAATGAAAAGGCTCTGGG  
CCGCAAAATAAACTCCTGGGAATCATCAAGGAGTGGGCATTTCCTTGAGCAACTTGCACTT  
GAGGAATGGTGAAGTGGTCATCCATGAAAAAGGGTTTTACTACATCTATTCCCAAACATACTT  
TCGATTTTCAGGAGG

10 SEQ ID NO:3

A 60 bp oligonucleotide probe with the following sequence was employed in the screening:

TGACGAAGAGAGTATGAACAGCCCCTGCTGGCAAGTCAAGTGGCAACTCCGTCAGCTCGT

SEQ ID NO:4

Hybridization was conducted overnight at room temperature in buffer containing 20% formamide, 5X SSC,  
15 10% dextran sulfate, 0.1% NaPiPO<sub>4</sub>, 0.05M NaPO<sub>4</sub>, 0.05 mg salmon sperm DNA, and 0.1% sodium dodecyl  
sulfate, followed by several washes at 42°C in 5X SSC, and then in 2X SSC. Twelve positive clones were  
identified in the cDNA library, and the positive clones were rescreened by hybridization to a second 60 bp  
oligonucleotide probe (not overlapping the first probe) having the following sequence:

GGTGAACTGGTCATCCATGAAAAAGGGTTTTACTACATCTATTCCCAAACATACTTTTGA

20 SEQ ID NO:5

Hybridization was conducted as described above.

Four resulting positive clones were identified and amplified by polymerase chain reaction  
(PCR) using a primer based on the flanking 5' vector sequence and adding an external ClaI restriction site and  
a primer based on the 3' flanking vector sequence and adding an external HindIII restriction site. PCR products  
25 were gel purified and subcloned into pGEM-T (commercially available from Promega) by T-A ligation. Three  
independent clones from different PCRs were then subjected to dideoxy DNA sequencing. DNA sequence  
analysis of these clones demonstrated that they were essentially identical, with some length variation at their  
5' region.

The nucleotide sequence of the coding region of Apo-2 ligand is shown in Figure 1A.  
30 Sequencing of the downstream 3' end region of one of the clones revealed a characteristic polyadenylation site  
(data not shown). The cDNA contained one long open reading frame with an initiation site assigned to the  
ATG codon at nucleotide positions 91-93. The surrounding sequence at this site is in reasonable agreement  
with the proposed consensus sequence for initiation sites [Kozak, *J. Cell. Biol.*, 115:887-903 (1991)]. The  
open reading frame ends at the termination codon TAA at nucleotide positions 934-936.

35 The predicted mature amino acid sequence of human Apo-2 ligand contains 281 amino acids,  
and has a calculated molecular weight of approximately 32.5 kDa and an isoelectric point of approximately  
7.63. There is no apparent signal sequence at the N-terminus, although hydropathy analysis (data not shown)

indicated the presence of a hydrophobic region between residues 15 and 40. The absence of a signal sequence and the presence of an internal hydrophobic region suggests that Apo-2 ligand is a type II transmembrane protein. The putative cytoplasmic, transmembrane and extracellular regions are 14, 26 and 241 amino acids long, respectively. The putative transmembrane region is underlined in Fig. 1A. A potential N-linked glycosylation site is located at residue 109 in the putative extracellular domain.

An alignment (using the Align<sup>TM</sup> computer program) of the amino acid sequence of the C-terminal region of Apo-2 ligand with other known members of the TNF cytokine family showed that, within the C-terminal region, Apo-2 ligand exhibits 23.2% identity to Apo-1 ligand (Figure 1B). The alignment analysis showed a lesser degree of identity with other TNF family members: CD40L (20.8%), LT- $\alpha$  (20.2%), LT- $\beta$  (19.6%), TNF- $\alpha$  (19.0%), CD30L and CD27L (15.5%), OX-40L (14.3%), and 4-1BBL (13.7%). In the TNF cytokine family, residues within regions which are predicted to form  $\beta$  strands, based on the crystal structures of TNF- $\alpha$  and LT- $\alpha$  [Eck et al., *J. Bio. Chem.*, 264:17595-17605 (1989); Eck et al., *J. Bio. Chem.*, 267:2119-2122 (1992)], tend to be more highly conserved with other TNF family members than are residues in the predicted connecting loops. It was found that Apo-2 ligand exhibits greater homology to other TNF family members in its putative  $\beta$  strand regions, as compared to homology in the predicted connecting loops. Also, the loop connecting putative  $\beta$  strands, B and B', is markedly longer in Apo-2 ligand.

## EXAMPLE 2

### Expression of Human Apo-2 Ligand

#### A. Full-length cDNA Fusion Construct

A full-length Apo-2 ligand cDNA fused to a myc epitope tag was constructed as follows. The Apo-2 ligand cDNA insert was excised from the parental pGEM-T Apo-2 ligand plasmid (described in Example 1) by digestion with ClaI and HindIII, and inserted into a pRK5 mammalian expression plasmid [Schall et al., *Cell*, 61:361-370 (1990); Suva et al., *Science*, 237:893-896 (1987)], which was digested with the same restriction enzymes. A sequence encoding a 13 amino acid myc epitope tag Ser Met Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn SEQ ID NO:6 [Evan et al., *Mol. Cell. Biol.*, 5:3610-3616 (1985)] was then inserted between codon 281 and the stop codon (codon 282) at the 3' end of the Apo-2 ligand coding sequence by oligonucleotide directed mutagenesis [Zoller et al., *Nucleic Acids Res.*, 10:6487-6496 (1982)] to give plasmid pRK5 Apo-2 ligand-myc.

The pRK5 Apo-2 ligand-myc plasmid was co-transfected into human 293 cells (ATCC CRL 1573) with a pRK5 plasmid carrying a neomycin resistance gene, by calcium phosphate precipitation. Stable clones expressing Apo-2 ligand-myc were selected by ability to grow in 50% HAM's F12/50% DMEM (GIBCO) media in the presence of the antibiotic, G418 (0.5 mg/mL) (GIBCO).

To investigate the topology of Apo-2 ligand, a G418-resistant clone was analyzed by FACS after staining with anti-myc monoclonal antibody (mAb) clone 9E10 [Evan et al., *supra*; commercially available from Oncogene Science) followed by a phycoerythrin (PE)-conjugated goat anti-mouse antibody (commercially available from Jackson ImmunoResearch). The FACS analysis revealed a specific positive staining shift in the Apo-2 ligand-myc-transfected clone as compared to mock transfected cells (Fig. 1C), showing that Apo-2 ligand is expressed at the cell-surface, with its carboxy terminus exposed. Accordingly, Apo-2 ligand is believed to be a type II transmembrane protein.

B. ECD Fusion Constructs

Two soluble Apo-2 ligand extracellular domain ("ECD") fusion constructs were prepared, in which another sequence was fused upstream of the C-terminal region of Apo-2 ligand.

In one construct, 27 amino acids of the herpes virus glycoprotein D ("gD") signal peptide  
 5 [described in Lasky et al., DNA, 3:23-29 (1984); Pennica et al., Proc. Natl. Acad. Sci., 92:1142-1146 (1995);  
 Paborsky et al., Protein Engineering, 3:547-553 (1990)] and epitope tag sequence

Lys Tyr Ala Leu Ala Asp Ala Ser Leu Lys Met Ala Asp Pro Asn Arg Phe Arg Gly Lys Asp Leu Pro Val Leu  
 Asp Gln

SEQ ID NO:7

10 were fused upstream to codons 114-281 of Apo-2 ligand within a pRK5 mammalian expression plasmid. Briefly, the gD sequence was amplified from a parent plasmid, pCHAD (Genentech, prepared substantially as described in Lasky et al., Science, 233:209-212 (1986)), in a PCR in which the 3' primer was complementary to the 3' region of the gD sequence as well as to codons 114-121 of Apo-2 ligand. The product was used as  
 15 a 5' primer along with a 3' primer complementary to the 3' end of the Apo-2 ligand-coding region in a subsequent PCR in which the pRK5 Apo-2 ligand plasmid was used as a template. The product, encoding the gD-Apo-2 ligand ECD fusion was then subcloned into a pRK5 plasmid to give the plasmid pRK5 gD-Apo-2 ligand ECD.

Human embryonic kidney 293 cells (ATCC CRL 1573) were transiently transfected with the pRK5 gD-Apo-2 ligand ECD plasmid or with pRK5, by calcium phosphate precipitation. Expression of  
 20 soluble gD-Apo-2 ligand protein was assessed by metabolic labeling of the transfected cells with <sup>35</sup>S-Cys and <sup>35</sup>S-Met. Cell supernatants were collected after 24 hours and cleared by centrifugation. For immunoprecipitation, 5 ml of supernatant were incubated with 5B6 anti-gD monoclonal antibody (Genentech) at 1 µg/ml overnight at 4°C. Then, 25 µl Pansorbin (Sigma) was added for another 1 hour at 4°C. The tubes were spun, the pellets were washed in PBS and boiled for 5 minutes in SDS sample buffer. The boiled samples  
 25 were spun again, and the supernatants were subjected to SDS-PAGE and autoradiography.

Immunoprecipitation with anti-gD antibody revealed three predominant protein bands in the supernatants of cells transfected with the gD-Apo-2 ligand plasmid (Fig. 1E). These bands migrated with relative molecular masses (*Mr*) of 23, 48 and 74 kDa. The calculated molecular weight of the mature gD-Apo-2 polypeptide is approximately 22.5 kDa; hence, the observed bands may represent monomeric (23 kDa),  
 30 dimeric (48 kDa) and trimeric (74 kDa) forms of the fusion protein, and indicate that Apo-2 ligand can be expressed as a secreted soluble gD fusion protein in mammalian cells.

In a second construct, a Met Gly His<sub>10</sub> sequence (derived from the plasmid pET19B, Novagen), followed by a 12 amino acid enterokinase cleavage site

Met Gly His His His His His His His His His Ser Ser Gly His Ile Asp Asp Asp Asp Lys His Met

35 SEQ ID NO:8

was fused upstream to codons 114-281 of Apo-2 ligand within a baculovirus expression plasmid (pVL1392, Pharmingen). Briefly, the Apo-2 ligand codon 114-281 region was amplified by PCR from the parent pRK5

Apo-2 ligand plasmid (described in Example 1) with primers complementary to the 5' and 3' regions which incorporate flanking NdeI and BamHI restriction sites respectively. The product was subcloned into pGEM-T (Promega) by T-A ligation, and the DNA sequence was confirmed. The insert was then excised by digestion with NdeI and BamHI and subcloned into a modified baculovirus expression vector pVL1392 (commercially available from Pharmingen) containing an amino terminal Met Gly His<sub>10</sub> tag and enterokinase cleavage site.

Recombinant baculovirus was generated by co-transfecting the His<sub>10</sub>-Apo-2 ECD plasmid and BaculoGold<sup>TM</sup> virus DNA (Pharmingen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses were harvested and used for further amplifications. Viral infection and protein expression was performed as described by O'Reilley et al., Baculovirus expression vectors: A laboratory Manual, Oxford:Oxford University Press (1994). The protein was purified by Ni<sup>2+</sup>-chelate affinity chromatography, as described in Example 3 below.

### EXAMPLE 3

#### Purification of Recombinant Human Apo-2 Ligand

Extracts were prepared from recombinant virus-infected and mock-infected Sf9 cells (see Example 2, section B above) as described by Rupert et al., *Nature*, 362:175-179 (1993). Briefly, Sf9 cells were washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM MgCl<sub>2</sub>; 0.1 mM EDTA; 10% Glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates were cleared by centrifugation, and the supernatant was diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% Glycerol, pH 7.8) and filtered through a 0.45 µm filter. A Ni<sup>2+</sup>-NTA agarose column (commercially available from Qiagen) was prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract was loaded onto the column at 0.5 mL per minute. The column was washed to baseline A<sub>280</sub> with loading buffer, at which point fraction collection was started. Next, the column was washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% Glycerol, pH 6.0), which eluted nonspecifically bound protein. After reaching A<sub>280</sub> baseline again, the column was developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions were collected and analyzed by SDS-PAGE and silver staining or western blot with Ni<sup>2+</sup>-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His<sub>10</sub>-Apo-2 ligand protein were pooled and dialyzed against loading buffer.

An identical procedure was repeated with mock-infected Sf9 cells as the starting material, and the same fractions were pooled, dialyzed, and used as control for the purified human Apo-2.

SDS-PAGE analysis of the purified protein revealed a predominant band of Mr 24 kDa, corresponding with the calculated molecular weight of 22.4 kDa for the His<sub>10</sub>-Apo-2 ligand monomer (Fig. 1D, lane 3); protein sequence microanalysis (data not shown) confirmed that the 24 kDa band represents the His<sub>10</sub>-Apo-2 ligand polypeptide. Minor 48 kDa and 66 kDa bands were also observed, and probably represent soluble Apo-2 ligand homodimers and homotrimers. Chemical crosslinking of the purified His<sub>10</sub>-Apo-2 ligand by incubation with sulfo-NHS (5mM) (Pierce Chemical) and EDC (Pierce Chemical) at 25 mM and 50 mM (Fig. 1D, lanes 1 and 2, respectively), shifted the protein into the 66 kDa band primarily. These results suggest

that the predominant form of Apo-2 ligand in solution is homotrimeric and that these trimers dissociate into dimers and monomers in the presence of SDS.

#### EXAMPLE 4

##### Apoptotic Activity of Apo-2 Ligand on Human Lymphoid Cell Lines

5 Apoptotic activity of purified, soluble Apo-2 ligand (described in Example 3) was examined using several human lymphoid cell lines. In a first study, the effect of Apo-2 ligand on 9D cells (Genentech, Inc.), derived from Epstein-Barr virus (EBV)-transformed human peripheral blood B cells, was examined. The 9D cells ( $5 \times 10^4$  cells/well in RPMI 1640 medium plus 10% fetal calf serum) were incubated for 24 hours with either a media control, Apo-2 ligand ( $3 \mu\text{g/ml}$ , prepared as described in Example 3 above), or anti-Apo-1  
10 monoclonal antibody, CH11 ( $1 \mu\text{g/ml}$ ) [described by Yonehara et al., *J. Exp. Med.*, 169:1747-1756 (1989); commercially available from Medical and Biological Laboratories Co.]. The CH11 anti-Apo-1 antibody is an agonistic antibody which mimicks Fas/Apo-1 ligand activity.

After the incubation, the cells were collected onto cytospin glass slides, and photographed under an inverted light microscope. Both Apo-2 ligand and the anti-Apo-1 monoclonal antibody induced a  
15 similar apoptotic effect, characterized by cytoplasmic condensation and reduction in cell numbers. (see Fig. 2A).

The effects of the Apo-2 ligand on the 9D cells, as well as on Raji cells (human Burkitt's lymphoma B cell line, ATCC CCL 86) and Jurkat cells (human acute T cell leukemia cell line, ATCC TIB 152) were further analyzed by FACS. The FACS analysis was conducted, using established criteria for apoptotic  
20 cell death, namely, the relation of fluorescence staining of the cells with two markers: (a) propidium iodide ("PI") dye, which stains apoptotic but not live cells, and (b) a fluorescent derivative of the protein, annexin V, which binds to the exposed phosphatidylserine found on the surface of apoptotic cells, but not on live cells [Darzynkiewicz et al., *Methods in Cell Biol.*, 41:15-38 (1994); Fadok et al., *J. Immunol.*, 148:2207-2214 (1992); Koopman et al., *Blood*, 84:1415-1420 (1994)].

25 The 9D cells (Fig. 2B), Raji cells (Fig. 2C), and Jurkat cells (Fig. 2D) were incubated ( $1 \times 10^6$  cells/well) for 24 hours with a media control (left panels), Apo-2 ligand ( $3 \mu\text{g/ml}$ , prepared as described in Example 3) (center panels), or anti-Apo-1 ligand antibody, CH11 ( $1 \mu\text{g/ml}$ ) (right panels). The cells were then washed, stained with PI and with fluorescein thiocyanate (FITC)-conjugated annexin V (purchased from Brand Applications) and analyzed by flow cytometry. Cells negative for both PI and annexin V staining  
30 (quadrant 3) represent live cells; PI-negative, annexin V-positive staining cells (quadrant 4) represent early apoptotic cells; PI-positive, annexin V-positive staining cells (quadrant 2) represent primarily cells in late stages of apoptosis.

The Apo-2 ligand treated 9D cells exhibited elevated extracellular annexin V binding, as well as a marked increase in uptake of PI (Fig. 2B), indicating that Apo-2 ligand induced apoptosis in the cells.  
35 Comparable results were obtained with anti-Apo-1 antibody, CH11 (Fig. 2B). The Apo-2 ligand induced a similar response in the Raji and Jurkat cells, as did the anti-Apo-1 antibody. (see Figures 2C and 2D). The induction of apoptosis (measured as the % apoptotic cells) in these cell lines by Apo-2 ligand, as compared to the control and to the anti-Apo-1 antibody, is also shown in Table 1 below.

The activation of internucleosomal DNA fragmentation by Apo-2 ligand was also analyzed. Jurkat cells (left lanes) and 9D cells (right lanes) were incubated ( $2 \times 10^6$  cells/well) for 6 hours with a media control or Apo-2 ligand ( $3 \mu\text{g/ml}$ , prepared as described in Example 3). The DNA was then extracted from the cells and labeled with  $^{32}\text{P}$ -ddATP using terminal transferase. The labeled DNA samples were subjected to electrophoresis on 2% agarose gels and later analyzed by autoradiography [Moore et al., *Cytotechnology*, 17:1-11 (1995)]. The Apo-2 ligand induced internucleosomal DNA fragmentation in both the Jurkat cells and 9D cells (Fig. 2E). Such DNA fragmentation is characteristic of apoptosis [Cohen, *Advances in Immunol.*, 50:55-85 (1991)].

To examine the time-course of the Apo-2 ligand apoptotic activity, 9D cells were incubated in microtiter dishes ( $5 \times 10^4$  cells/well) with a media control or Apo-2 ligand ( $3 \mu\text{g/ml}$ , prepared as described in Example 3) for a period of time ranging from 0 hours to 50 hours. Following the incubation, the numbers of dead and live cells were determined by microscopic examination using a hemocytometer.

As shown in Fig. 3A, maximal levels of cell death were induced in 9D cells within 24 hours.

To determine dose-dependency of Apo-2 ligand-induced cell death, 9D cells were incubated ( $5 \times 10^4$  cells/well) for 24 hours with serial dilutions of a media control or Apo-2 ligand (prepared as described in Example 3). The numbers of dead and live cells following the incubation were determined as described above. The results are illustrated in Fig. 3B. Specific apoptosis was determined by subtracting the % apoptosis in the control from % apoptosis in Apo-2 ligand treated cells. Half-maximal activation of apoptosis occurred at approximately  $0.1 \mu\text{g/ml}$  (approximately 1 nM), and maximal induction occurred at about 1 to about  $3 \mu\text{g/ml}$  (approximately 10 to 30 nM).

#### EXAMPLE 5

##### Apoptotic Activity of Apo-2 Ligand on Human Non-lymphoid

##### Tumor Cell Lines

The effect of Apo-2 ligand on human non-lymphoid tumor cell lines was examined using the following cell lines: HeLa (derived from human cervical carcinoma, ATCC CCL 22); ME-180 (derived from human cervical carcinoma, ATCC HTB 33); MCF7 (derived from human breast carcinoma, ATCC HTB 22); U-937 (derived from human histiocytic lymphoma, ATCC CRL 1593); A549 (derived from human lung carcinoma, ATCC CCL 185); and 293 (derived from an adenovirus-transformed human embryonic kidney cells, ATCC CCL 1573).

In the assay,  $1 \times 10^6$  cells of each cell line were incubated for 24 hours with a media control, Apo-2 ligand ( $3 \mu\text{g/ml}$ , prepared as described in Example 3), or anti-Apo-1 monoclonal antibody, CH 11 ( $1 \mu\text{g/ml}$ ). Following the incubation, apoptosis was measured by FACS analysis, as described in Example 4. The results are shown below in Table 1.

TABLE I

Cell line	% apoptotic cells		
	Control	Apo-2L	Anti-Apo-1 Ab
<u>Lymphoid</u>			
9D	22.5	92.4	90.8
Raji	35.9	73.4	83.7
Jurkat	5.9	77.0	18.1
<u>Non-lymphoid</u>			
HeLa	5.3	18.6	17.9
MCF7	39.9	47.3	44.0
U-937	3.6	62.3	16.6
A549	16.5	74.6	25.1
ME-180	8.6	80.7	9.9
293	12.3	12.2	16.7

The HeLa cells and MCF7 cells were equally sensitive to induction of apoptosis by Apo-2 ligand as compared to the CH11 anti-Apo-1 antibody. In contrast, the U-937 cells and A549 cells were markedly more sensitive to induction of apoptosis by Apo-2 ligand. The ME-180 cells were quite sensitive to the Apo-2 ligand, but were relatively resistant to the anti-apo-1 antibody. The 293 cells were resistant to the Apo-2 ligand and weakly responsive to the anti-Apo-1 antibody.

Thus, Apo-2 ligand is capable of inducing apoptosis in cells of non-lymphoid origin, as well as cells of lymphoid origin (see Example 4). Also, although not fully understood and not wishing to be bound by any particular theory, Applicants presently believe that Apo-2 ligand acts via a receptor which is distinct from Apo-1. This belief is supported by the data herein showing that the cell lines described above exhibit differential patterns of sensitivity to Apo-2 ligand and to anti-Apo-1 antibody. (see also, Example 7 below).

#### EXAMPLE 6

##### Effect of Apo-2 Ligand on Human Peripheral Blood Monocytes

Peripheral blood mononuclear cells ("PBMC") were isolated from the blood of human donors by Ficoll density gradient centrifugation using Lymphocyte Separation Medium (LSM®, Organon Teknika). An isolated population of T cells was prepared from the PBMC by removal of B cells through surface Ig binding to an anti-Ig column and removal of monocytes through Fc receptor binding to an Ig column (R & D Systems). An isolated population of B cells was prepared from the PBMC by complement-mediated elimination of T cells reacted with the anti-CD3 antibody produced by the OKT3 myeloma (ATCC, CRL 8001)



and of monocytes reacted with a monocyte-specific antibody produced by the 4F2C13 hybridoma (ATCC, HB 22). Additional monocyte removal was accomplished by adherence to plastic.

The freshly isolated peripheral blood B or T cells ( $1 \times 10^6$  cells/well) were cultured for 3 days in the presence of a media control or Apo-2 ligand ( $3 \mu\text{g/ml}$ , prepared as described in Example 3). For activation, B cells were treated simultaneously with lipopolysaccharide ("LPS",  $1 \mu\text{g/ml}$ ), and T cells were treated with phorbol myristate acetate ("PMA",  $10 \text{ ng/ml}$ ) plus ionomycin ( $1 \mu\text{g/ml}$ ) (Sigma). For interleukin-2 ("IL-2") pretreatment, T cells were cultured for 3-5 days in the presence of IL-2 ( $50 \text{ U/ml}$ ) (Genzyme) before exposure to Apo-2 ligand. Apoptosis was determined using FACS analysis essentially as described above in Example 4. However, B cells were gated by anti-CD19/CD20 antibodies (Jackson Immunoresearch), and T cells were gated by anti-CD4/CD8 antibodies (Jackson Immunoresearch). The results are shown in Table 2 below, representing means  $\pm$  SE of independent experiments [B lymphocytes - 9 experiments; T lymphocytes - 8 experiments; T lymphocytes plus IL-2 - 5 experiments], in which 50,000 cells were analyzed per data point. Statistical analysis was performed using the student t-test. In Table 2,  $a=p<0.05$  and  $b=p<0.02$  relative to the respective control.

TABLE 2

Treatment		% apoptotic cells	
		Control	Apo-2L
<u>B lymphocytes</u>	none	$40.1 \pm 4.1$	$53.2 \pm 3.3^a$
	LPS	$44.8 \pm 2.8$	$55.9 \pm 3.2^a$
<u>T lymphocytes</u>	none	$6.3 \pm 0.6$	$8.2 \pm 0.8$
	PMA/ionomycin	$40.3 \pm 4.4$	$54.2 \pm 3.3^a$
	IL-2 pretreatment	$13.7 \pm 1.2$	$34.5 \pm 4.8^b$

Apo-2 ligand induced significant apoptosis in unstimulated B cells, in B cells activated by LPS and in T cells activated with PMA and ionomycin. It was previously reported that peripheral T cells can be predisposed to apoptosis by culturing the cells in the presence of IL-2 [Lenardo et al., *Nature*, 353:858-861 (1991)]. The present study showed that pretreatment with IL-2 did sensitize the peripheral T cells to Apo-2 ligand-induced death.

#### EXAMPLE 7

##### Inhibition Assay Using Fas/Apo-1 and TNF Receptors

An assay was conducted to determine if the Fas/Apo-1 receptor, as well as the type 1 and type 2 TNF receptors (TNF-R1 and TNF-R2), are involved in mediating the apoptotic activity of Apo-2 ligand by

testing if soluble forms of these receptors are capable of inhibiting the apoptotic activity of purified, soluble Apo-2 ligand (described in Example 3).

9D cells were incubated ( $5 \times 10^4$  cells/well) for 24 hours with a media control or Apo-2 ligand (0.3  $\mu\text{g/ml}$ , prepared as described in Example 3) in the presence of buffer control, CD4-IgG control (25  $\mu\text{g/ml}$ ), soluble Apo-1-IgG (25  $\mu\text{g/ml}$ ), soluble TNFR1-IgG (25  $\mu\text{g/ml}$ ) or soluble TNFR2-IgG fusion protein (25  $\mu\text{g/ml}$ ). Soluble derivatives of the Fas/Apo-1, TNF-R1 and TNF-R2 receptors were produced as IgG fusion proteins as described in Ashkenazi et al., *Methods*, 8:104-115 (1995). CD4-IgG was produced as an IgG fusion protein as described in Byrn et al., *Nature*, 344:667-670 (1990) and used as a control.

As shown in Figure 3C, none of the receptor-fusion molecules inhibited Apo-2 ligand apoptotic activity on the 9D cells. These results indicate that Apo-2 ligand apoptotic activity is independent of Fas/Apo-1 and of TNF-R1 and TNF-R2.

### EXAMPLE 8

#### Expression of Apo-2 Ligand mRNA in Mammalian Tissues

Expression of Apo-2 ligand mRNA in human tissues was examined by Northern blot analysis (Fig. 4). Human RNA blots were hybridized to a  $^{32}\text{P}$ -labeled DNA probe based on the full-length Apo-2 ligand cDNA, or to a  $^{32}\text{P}$ -labeled RNA probe based on the GenBank EST sequence, HHEA47M (see Example 1). Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probe, while human adult RNA blot MTN-I (Clontech) was incubated with the RNA probe. Blots were incubated with the probes in hybridization buffer (5 X SSPE; 2 X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 16 hours at  $42^\circ\text{C}$ . The blots were washed several times in 1 X SSPE; 2% SDS for 1 hour at  $65^\circ\text{C}$  and 50% freshly deionized formamide; 1 X SSPE; 0.2% SDS for 30 minutes at  $65^\circ\text{C}$ . The blots were developed after overnight exposure, using a phosphorimager (Fuji).

The results are shown in Figure 4. In fetal human tissues, Apo-2 ligand mRNA expression was detected in lung, liver and kidney, but not in brain tissue. In adult human tissues, Apo-2 ligand mRNA expression was detected in spleen, thymus, prostate, ovary, small intestine, peripheral blood lymphocytes, heart, placenta, lung, and kidney. Little or no expression was detected in testis, brain, skeletal muscle, and pancreas. The expression profile observed for Apo-2 ligand, as described above, is not identical to that of Apo-1 ligand, which is expressed primarily in T cells and testis [Nagata et al., *supra*].

### EXAMPLE 9

#### Apoptotic Activity of Apo-2 Ligand on Human Tumor Cell Lines

Apoptotic activity of Apo-2 ligand (described in Example 3) on human tumor cell lines was further examined in the presence or absence of one of several chemotherapeutic agents.

The following human tumor cell lines were assayed: A549 (lung carcinoma, ATCC CCL 185); HCT116 (colon carcinoma, ATCC CCL 247); SW480 (colon adenocarcinoma, ATCC CCL 228); MDA231 (breast adenocarcinoma, ATCC HTB 26); HeLa (cervical carcinoma, ATCC CCL 22); ME-180 (cervical carcinoma, ATCC HTB 33); T24 (bladder carcinoma, ATCC HTB 4); SK-N-AS (neuroblastoma, White et al., *Proc. Natl. Acad. Sci.*, 92:5520-5524 (1995)). Several of these cell lines express wild-type p53 while the others do not due to mutations, as shown in Table 3 below. The cells were plated at  $2.5 \times 10^5$

cells/ml in 96 well plates and incubated overnight. The cells were cultured in the presence of 2-fold dilutions of Apo-2 ligand (ranging from 100 ng/ml to 0.01 ng/ml). In some of the cultures, a chemotherapeutic agent was also added for 24 hours- cyclohexamide ("CHX") (50 µg/ml; Sigma Chemicals), Doxorubicin (10-100 µg/ml; Pharmacia), or 5-FU (6 mg/ml; Roche).

- 5 After 24 hours of incubation, the cells were stained with 0.5% crystal-violet in 20% methanol. Cell viability was determined by eluting the dye from the stained cells with 0.1M sodium citrate (0.1M citric acid in 50% methanol), and measuring absorbance at 540 nm.

The results are shown in the Table below.

TABLE 3

CELL LINE	TUMOR TYPE	p53 STATUS	Apo-2L SENSITIVITY	ENHANCED BY		
				CHX	Dox	5-FU
A549	lung carcinoma	WT	++	yes	yes	yes
HCT116	colon carcinoma	mut	++	yes	yes	yes
SW480	colon carcinoma	mut	++	yes	yes	yes
MDA231	breast carcinoma	mut	++	yes	yes	yes
15 HeLa	cervical carcinoma	mut	+	yes	ND	ND
ME180	cervical carcinoma	WT	++	yes	yes	ND
T24	bladder carcinoma	mut	+++	yes	yes	ND
SK-N-AS	neuroblastoma	?	+	ND	ND	ND

- 20 ++: >35% death after 24h with 100 ng/ml Apo-2L  
 +++: >70% death after 24h with 100 ng/ml Apo-2L  
 ++++: >70% death after 24h with 10 ng/ml Apo-2L

These results show that Apo-2 ligand induced cell death in tumor cell lines derived from various tumor types and that Apo-2L induced cell death independently of the p53 status of the tumor cells. These results also show that the Apo-2 ligand-induced cell death is augmented by several different chemotherapeutic drugs.

#### 25 EXAMPLE 10

##### Apoptotic Activity of Apo-2 Ligand *in vivo*

- The effects of Apo-2 ligand were examined in tumor bearing nude mice. Nude mice (5-10 mice per group) (purchased from Harlan Sprague Dawley) were injected (Day 0) subcutaneously with MDA231 human breast carcinoma cells (ATCC HTB 26) ( $2 \times 10^6$  cells/mouse). The tumors were then allowed to grow for 14 days. On Days 14 and 15, 2 µg/0.05ml/mouse Apo-2 ligand (Example 3) and/or 10 µg/0.05ml/mouse Doxorubicin (Pharmacia) was injected at the tumor site. Control animals were similarly injected with 0.05ml PBS. On Day 21, the animals were sacrificed and the tumors excised and weighed (grams).

The results are shown in Figure 5. The data shows that Apo-2 ligand treatment inhibited tumor growth by itself and that Apo-2 ligand enhanced the inhibitory effects of Doxorubicin on tumor growth.

### EXAMPLE 11

#### Apoptotic Activity of Apo-2 Ligand *in vivo*

The anti-tumor effects of Apo-2 ligand were also examined in tumor bearing nude mice, as described in Example 10, except that on Day 0, the mice were injected subcutaneously with HCT116 human colon carcinoma cells (ATCC CCL 247) ( $2 \times 10^6$  cells/mouse). The tumors were then allowed to grow for 14 days. On Days 14 and 15, 2  $\mu\text{g}/0.05\text{ml}/\text{mouse}$  Apo-2 ligand and/or 10  $\mu\text{g}/0.05\text{ml}/\text{mouse}$  5-FU (Roche) was injected at the tumor site. Control animals were similarly injected with 0.05ml PBS. On Day 21, the animals were sacrificed and the tumors excised and weighed (grams).

The results are shown in Figure 6. These results show that Apo-2 ligand treatment inhibited tumor growth by itself and that Apo-2 ligand enhanced the inhibitory effects of 5-FU on tumor growth.

### EXAMPLE 12

#### Apoptotic Activity of Apo-2 Ligand *in vivo*

The anti-tumor effects of Apo-2 ligand were examined in tumor bearing nude mice, as described in Example 11, except that on Days 1 and 2, 10  $\mu\text{g}/0.05\text{ml}/\text{mouse}$  Apo-2 ligand and/or 100  $\mu\text{g}/0.05\text{ml}/\text{mouse}$  5-FU was injected intraperitoneally. Control animals were similarly injected with PBS. Tumor size ( $\text{mm}^2$ ) was then measured on Days 5, 9, and 15. On Day 15, the animals were sacrificed and the tumors excised and weighed (grams).

The results are shown in Figures 7 and 8. These results show that Apo-2 ligand is capable of reaching the subcutaneous tumor site and exerting an anti-tumor effect even when administered by intraperitoneal injection. Also, these results confirm the ability of Apo-2 ligand treatment to inhibit tumor growth by itself and to enhance the inhibitory effects of 5-FU on tumor growth.

### EXAMPLE 13

#### Inhibition Assay Using CrmA

To investigate whether proteases such as ICE and CPP32/Yama play a role in apoptosis-induction by Apo-2 ligand, an assay was conducted to determine if CrmA blocks Apo-2 ligand-induced apoptosis [Marsters et al., Current Biology, 6:750-752 (1996)]. CrmA is a poxvirus-derived inhibitor of the death proteases ICE and CPP32/Yama and blocks death signalling by TNFR1 and Fas/Apo-1. In addition, to investigate if the "death domain" containing adaptor protein, FADD, which mediates apoptosis induction by Apo-1 ligand and by TNF [Chinnaiyan et al., Cell, 81:505-512 (1995); Hsu et al., Cell, 84:299-308 (1996)], is involved in Apo-2 ligand-induced apoptosis, an assay was conducted to determine if a dominant negative mutant form of FADD, (FADD-DN) [Hsu et al., supra], inhibits Apo-2 ligand function [Marsters et al., Current Biology, 6:750-752 (1996)].

HeLa-S3 (ATCC CCL 22) cells were transfected with a pRK5-CrmA expression plasmid (CrmA sequence reported in Ray et al., supra) or a pRK-5-FADD-DN expression plasmid (Hsu et al., Cell, 84:299-308 (1996)). pRK5 was used as a control. The cells were co-transfected with pRK5-CD4 (Smith et al., Science, 228:1704-1707 (1988)) as a marker for uptake of plasmid DNA. Transfected cells were identified by staining with phycoerythrin-conjugated anti-CD4 antibody (Jackson Immunoresearch) and apoptosis was analyzed by FACS essentially as described in Example 4 above.

The results are shown in Figure 9. CrmA blocked Apo-2 ligand-induced apoptosis, as well as apoptosis induced by anti-Apo-1 antibody. In contrast, FADD-DN had little effect on Apo-2 ligand-induced apoptosis but blocked substantially the apoptosis induction by anti-Apo-1 antibody. Accordingly, the assay results suggest that Apo-2 ligand, TNFR1 and Fas/Apo-1 may engage a common distal signalling pathway to activate apoptotic cell death. In particular, the results suggest that proteases such as ICE and CPP32/Yama may be required for Apo-2 ligand induced apoptosis. In contrast, FADD is required for cell death induction by TNFR1 and Fas/Apo-1, but not by Apo-2 ligand.

#### EXAMPLE 14

##### A. Preparation of anti-Apo-2 Ligand Antibodies

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 1  $\mu$ g Apo-2 ligand (prepared as described in Example 3 and diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) ten times into each hind foot pad at 1 week intervals. Three days after the final boost injection, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol [Laskor et al., *Cell Immunol.*, 55:251 (1980)] and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA [Kim et al., *J. Immunol. Meth.*, 156:9-17 (1992)] to test for the presence of monoclonal antibodies binding to the Apo-2 ligand protein.

In the ELISA, 96-well microtiter plates (Nunc) were coated by adding 50  $\mu$ l of 0.5  $\mu$ g/ml Apo-2 ligand (see Example 3) in PBS to each well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS plus 0.05% Tween 20). The wells in the microtiter plates were then blocked with 200  $\mu$ l of 2% bovine serum albumin (BSA) and incubated at room temperature for 1 hour. The plates were then washed again three times with wash buffer.

After the washing step, 50  $\mu$ l of 2  $\mu$ g/ml of the Apo-2 ligand antibodies or 100  $\mu$ l of the hybridoma culture supernatant was added to designated wells. 100  $\mu$ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50  $\mu$ l HRP-conjugated goat anti-mouse IgG (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50  $\mu$ l of substrate (TMB, 3,3',5,5'-tetramethylbenzidine; obtained from Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50  $\mu$ l of stop solution (Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Hybridoma supernatants (99 selected) were tested for activity to block Apo-2 ligand-induced 9D cell killing. Activity was initially determined by examining % viability of treated 9D cells using trypan blue dye exclusion.

Blocking activity was also confirmed by FACS analysis. The 9D cells ( $5 \times 10^5$  cells/0.5ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate) and placed into 24-well macro-titer plates. 0.5 ml of Apo-2 ligand ( $1 \mu\text{g/ml}$ ) (prepared as described in Example 3) was suspended into complete RPMI media, preincubated with 10  $\mu\text{g}$  of purified monoclonal antibodies or 100  $\mu\text{l}$  of culture supernatant, and then added into the 24 macro-titer wells containing 9D cells. The macro-titer plates were incubated overnight at  $37^\circ\text{C}$  and in the presence of 7%  $\text{CO}_2$ . The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200  $\mu\text{l}$  binding buffer. Ten  $\mu\text{l}$  of annexin-V-FITC ( $1 \mu\text{g/ml}$ ) and 10  $\mu\text{l}$  of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

Eight potential blocking and 4 potential non-blocking antibody secreting hybridomas were identified and were further cloned (twice) by limiting dilution techniques.

FACS analysis of four antibodies, referred to as monoclonal antibodies 1D1, 2G6, 2E11, and 5C2, is illustrated in Figure 10 (As indicated below, the 1D1, 2G6, 2E11 and 5C2 antibodies are produced by hybridomas 1D1.12.4, 2G6.3.4, 2E11.5.5, and 5C2.4.9, respectively, all of which have been deposited with the ATCC). The 9D cells treated with the Apo-2 ligand (top left figure) showed 50% apoptotic cells above the untreated, control cells (top right figure). The 9D cells treated with Apo-2 ligand plus the 2E11, 5C2, 2G6, or 1D1 antibodies showed 0%, 6%, 26%, and 48% apoptotic cells above the untreated control, respectively. These results show that the 5C2, 2E11 and 2G6 antibodies are blocking antibodies while the 1D1 antibody is a non-blocking antibody. The most potent blocking activity was observed with the 5C2 antibody.

The antigen specificities of the four antibodies was also tested in an ELISA. Microtiter wells were coated with 2  $\mu\text{g/ml}$  lymphotoxin (Genentech, Inc., see also, EP 164,965, Gray et al., *Nature*, 312:721-724 (1984)), TNF-alpha (Genentech, Inc., see also, Pennica et al., *Nature*, 312:724-729 (1984); Aggarwal et al., *J. Biol. Chem.*, 260:2345-2354 (1985)), or Apo-2 ligand (see Example 3). Monoclonal antibodies 1D1, 2G6, 2E11, and 5C2 were tested at a concentration of 10  $\mu\text{g/ml}$ .

The results of the assay are shown in Figure 11. The data in Figure 11 shows that monoclonal antibodies 2G6, 2E11 and 5C2 are specific for Apo-2 ligand, while monoclonal antibody 1D1 showed weak cross-reactive binding to lymphotoxin and to TNF-alpha.

#### 30 B. Isotyping

The isotypes of the 1D1, 2G6, 2E11, and 5C2 antibodies (described in Section A above) were determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at  $4^\circ\text{C}$ . The plates were then washed with wash buffer as described above. The wells in the microtiter plates were then blocked with 200  $\mu\text{l}$  of 2% bovine serum albumin and incubated at room temperature for 1 hour. The plates were washed again three times with wash buffer.

Next, 100  $\mu\text{l}$  of 5  $\mu\text{g/ml}$  of the purified Apo-2 ligand antibodies or 100  $\mu\text{l}$  of the hybridoma culture supernatant was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50  $\mu\text{l}$  HRP-conjugated goat anti-mouse IgG (as described above) was added to each well.

The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

The isotyping analysis showed that the 1D1 and 2G6 antibodies are IgG2b antibodies, and the 2E11 and 5C2 antibodies are IgG2a antibodies.

5 C. Epitope mapping

Epitope mapping was performed using a competitive binding ELISA as described in Kim et al., *supra*, using biotinylated monoclonal antibodies. The selected monoclonal antibodies were biotinylated using N-hydroxysuccinimide as described in Antibodies, A laboratory Manual, Eds. E. Harlow and D. Lane, p. 342. Microtiter plate wells were coated with 50  $\mu$ l of Apo-2 ligand (see Example 3, 0.1  $\mu$ g/ml) and kept  
 10 overnight at 4°C, and then blocked with 2% BSA for 1 hour at room temperature. After washing the microtiter wells, a mixture of a predetermined optimal concentration of biotinylated antibodies and a thousand-fold excess of unlabeled antibody was added into each well. Following a 1 hour incubation at room temperature, the plates were washed and the amount of biotinylated antibody was detected by the addition of HRP-streptavidin. After washing the microtiter wells, the bound enzyme was detected by the addition of the substrate (TMB), and the  
 15 plates were read at 490 nm with an ELISA plate reader.

The results are shown in Figure 12. The results show that the binding of the HRP-conjugated antibodies was effectively inhibited by the excess amount of its own antibody but not by the other antibodies assayed.

The regions of the Apo-2 ligand recognized by the monoclonal antibodies were determined  
 20 using synthetic peptides [aa 128-143 (peptide "APO 14"); aa 144-159 (peptide "APO 15"); aa 192-204 (peptide "APO 17"); aa 230-238 (peptide "APO 18"); aa 261-272 (peptide "APO 19") of the Apo-2 ligand sequence as shown in Fig. 1A] in an ELISA as described in Chuntharapai et al., J. Immunol., 152:1783-1789 (1994). The results are shown in Figure 13. The 1D1 antibodies showed binding to the APO 17 peptide comprising amino acid residues 192-204 of Apo-2 ligand.

25 \* \* \* \* \*

Deposit of Material

The following cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Cell line</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
30 2935-pRK5-hApo-2L-myc clone 2.1	CRL-12014	Jan.3,1996
1D1.12.4	_____	_____
2G6.3.4	_____	_____
2E11.5.5	_____	_____
35 5C2.4.9	_____	_____

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and  
5 subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR  
10 §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the cell line on deposit should die or be lost or destroyed when cultivated under suitable conditions, the cell line will be promptly replaced on notification with another of the same plasmid. Availability of the deposited cell line is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any  
15 government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein  
20 does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
- (ii) TITLE OF INVENTION: Apo-2 Ligand
- 5 (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Genentech, Inc.
  - (B) STREET: 460 Point San Bruno Blvd
  - (C) CITY: South San Francisco
  - 10 (D) STATE: California
  - (E) COUNTRY: USA
  - (F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
  - 15 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
  - 20 (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/584031
  - (B) FILING DATE: 09-JAN-1996
- 25 (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Marschang, Diane L.
  - (B) REGISTRATION NUMBER: 35,600
  - (C) REFERENCE/DOCKET NUMBER: P0978P1PCT
- (ix) TELECOMMUNICATION INFORMATION:
  - 30 (A) TELEPHONE: 415/225-5416
  - (B) TELEFAX: 415/952-9881
  - (C) TELEX: 910/371-7168

## (2) INFORMATION FOR SEQ ID NO:1:

- 35 (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 281 amino acids
  - (B) TYPE: Amino Acid
  - (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
 

40	Met	Ala	Met	Met	Glu	Val	Gln	Gly	Gly	Pro	Ser	Leu	Gly	Gln	Thr	
	1				5					10					15	
	Cys	Val	Leu	Ile	Val	Ile	Phe	Thr	Val	Leu	Leu	Gln	Ser	Leu	Cys	
				20						25					30	
	Val	Ala	Val	Thr	Tyr	Val	Tyr	Phe	Thr	Asn	Glu	Leu	Lys	Gln	Met	
				35						40					45	
45	Gln	Asp	Lys	Tyr	Ser	Lys	Ser	Gly	Ile	Ala	Cys	Phe	Leu	Lys	Glu	
				50						55					60	
	Asp	Asp	Ser	Tyr	Trp	Asp	Pro	Asn	Asp	Glu	Glu	Ser	Met	Asn	Ser	
				65						70					75	

	Pro Cys Trp Gln Val Lys Trp Gln Leu Arg Gln Leu Val Arg Lys	80	85	90
	Met Ile Leu Arg Thr Ser Glu Glu Thr Ile Ser Thr Val Gln Glu	95	100	105
5	Lys Gln Gln Asn Ile Ser Pro Leu Val Arg Glu Arg Gly Pro Gln	110	115	120
	Arg Val Ala Ala His Ile Thr Gly Thr Arg Gly Arg Ser Asn Thr	125	130	135
10	Leu Ser Ser Pro Asn Ser Lys Asn Glu Lys Ala Leu Gly Arg Lys	140	145	150
	Ile Asn Ser Trp Glu Ser Ser Arg Ser Gly His Ser Phe Leu Ser	155	160	165
	Asn Leu His Leu Arg Asn Gly Glu Leu Val Ile His Glu Lys Gly	170	175	180
15	Phe Tyr Tyr Ile Tyr Ser Gln Thr Tyr Phe Arg Phe Gln Glu Glu	185	190	195
	Ile Lys Glu Asn Thr Lys Asn Asp Lys Gln Met Val Gln Tyr Ile	200	205	210
20	Tyr Lys Tyr Thr Ser Tyr Pro Asp Pro Ile Leu Leu Met Lys Ser	215	220	225
	Ala Arg Asn Ser Cys Trp Ser Lys Asp Ala Glu Tyr Gly Leu Tyr	230	235	240
	Ser Ile Tyr Gln Gly Gly Ile Phe Glu Leu Lys Glu Asn Asp Arg	245	250	255
25	Ile Phe Val Ser Val Thr Asn Glu His Leu Ile Asp Met Asp His	260	265	270
	Glu Ala Ser Phe Phe Gly Ala Phe Leu Val Gly	275	280	281

## (2) INFORMATION FOR SEQ ID NO:2:

30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1042 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTCCTCACT GACTATAAAA GAATAGAGAA GGAAGGGCTT CAGTGACCGG 50  
 CTGCCTGGCT GACTTACAGC AGTCAGACTC TGACAGGATC ATGGCTATGA 100  
 TGGAGGTCCA GGGGGGACCC AGCCTGGGAC AGACCTGCGT GCTGATCGTG 150  
 ATCTTCACAG TGCTCCTGCA GTCTCTCTGT GTGGCTGTAA CTTACGTGTA 200  
 40 CTTTACCAAC GAGCTGAAGC AGATGCAGGA CAAGTACTCC AAAAGTGGCA 250  
 TTGCTTGT TTCTTAAAGAA GATGACAGTT ATTGGGACCC CAATGACGAA 300  
 GAGAGTATGA ACAGCCCCTG CTGGCAAGTC AAGTGGCAAC TCCGTCAGCT 350

CGTTAGAAAG ATGATTTTGA GAACCTCTGA GGAAACCATT TCTACAGTTC 400  
 AAGAAAAGCA ACAAATATT TCTCCCCTAG TGAGAGAAAG AGGTCCTCAG 450  
 AGAGTAGCAG CTCACATAAC TGGGACCAGA GGAAGAAGCA ACACATTGTC 500  
 TTCTCCAAAC TCCAAGAATG AAAAGGCTCT GGGCCGCAA ATAAACTCCT 550  
 5 GGGAAATCATC AAGGAGTGGG CATTCAATTCC TGAGCAACTT GCACTTGAGG 600  
 AATGGTGAAC TGGTCATCCA TGAAAAAGGG TTTTACTACA TCTATTCCCA 650  
 AACATACTTT CGATTTCAGG AGGAAATAAA AGAAAACACA AAGAACGACA 700  
 AACAAATGGT CCAATATATT TACAAATACA CAAGTTATCC TGACCCTATA 750  
 TTGTTGATGA AAAGTGCTAG AAATAGTTGT TGGTCTAAAG ATGCAGAATA 800  
 10 TGGACTCTAT TCCATCTATC AAGGGGGAAT ATTTGAGCTT AAGGAAAATG 850  
 ACAGAATTTT TGTTCCTGTA ACAAATGAGC ACTTGATAGA CATGGACCAT 900  
 GAAGCCAGTT TTTTCGGGGC CTTTTAGTT GGCTAACTGA CCTGGAAAGA 950  
 AAAAGCAATA ACCTCAAAGT GACTATTCAG TTTTCAGGAT GATACACTAT 1000  
 GAAGATGTTT CAAAAATCT GACCAAAACA AACAAACAGA AA 1042

15 (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 390 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 20 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGACCCCAA TGACGAAGAG AGTATGAACA GCCCCTGCTG GCAAGTCAAG 50  
 TGGCAACTCC GTCAGCTCGT TAGAAAGATG ATTTTGAGAA CCTCTGAGGA 100  
 AACCATTTCT ACAGTTCAAG AAAAGCAACA AAATATTTCT CCCCTAGTGA 150  
 25 GAGAAAGAGG TCCTCAGAGA GTAGCAGCTC ACATAACTGG GACCAGAGGA 200  
 AGAAGCAACA CATTGTCTTC TCCAACTCC AAGAATGAAA AGGCTCTGGG 250  
 CCGCAAATA AACTCCTGGG AATCATCAAG GAGTGGGCAT TCATTCCTGA 300  
 GCAACTTGCA CTTGAGGAAT GGTGAAGTGG TCATCCATGA AAAAGGGTTT 350  
 TACTACATCT ATTCCCAAAC ATACTTTCGA TTTCAGGAGG 390

30 (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 60 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 35 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGACGAAGAG AGTATGAACA GCCCCTGCTG GCAAGTCAAG TGGCAACTCC 50  
 GTCAGCTCGT 60

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 5 (A) LENGTH: 60 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10 GGTGAACTGG TCATCCATGA AAAAGGGTTT TACTACATCT ATTCCCAAAC 50  
 ATACTTTCGA 60

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 15 (A) LENGTH: 13 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Met Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn  
 1 5 10 13

## 20 (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 27 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Tyr Ala Leu Ala Asp Ala Ser Leu Lys Met Ala Asp Pro Asn  
 1 5 10 15  
 Arg Phe Arg Gly Lys Asp Leu Pro Val Leu Asp Gln  
 20 25 27

## 30 (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly His His His His His His His His His Ser Ser Gly  
 1 5 10 15  
 His Ile Asp Asp Asp Asp Lys His Met  
 20 24

## WHAT IS CLAIMED IS:

1. Isolated biologically active Apo-2 ligand comprising amino acid residues 114-281 of Figure 1A.
2. The Apo-2 ligand of claim 1 comprising amino acid residues 41-281 of Figure 1A.
- 5 3. The Apo-2 ligand of claim 2 comprising amino acid residues 15-281 of Figure 1A.
4. The Apo-2 ligand of claim 3 comprising amino acid residues 1-281 of Figure 1A.
5. Isolated biologically active Apo-2 ligand having amino acid residues 1-281 of Figure 1A.
6. Isolated biologically active Apo-2 ligand having at least about 80% sequence identity with either of:
  - 10 (a) the full-length native human Apo-2 ligand comprising amino acid residues 1-281 of Figure 1A;
  - (b) the extracellular region of native human Apo-2 ligand comprising amino acid residues 41-281 of Figure 1A; or
  - (c) the extracellular region of native human Apo-2 ligand comprising amino acid residues 114-281 of Figure 1A.
7. The Apo-2 ligand of claim 6 wherein said ligand has at least about 90% sequence identity with either of (a), (b), or (c).
8. The Apo-2 ligand of claim 7 wherein said ligand has at least about 95% sequence identity with either of (a), (b), or (c).
- 20 9. A chimeric polypeptide comprising the Apo-2 ligand of claim 1 fused to a heterologous polypeptide sequence.
10. The chimeric polypeptide of claim 9 wherein said heterologous polypeptide sequence is a tag polypeptide sequence.
11. Isolated nucleic acid encoding Apo-2 ligand.
- 25 12. The nucleic acid of claim 11 wherein said nucleic acid encodes Apo-2 ligand comprising amino acid residues 114-281 of Figure 1A.
13. The nucleic acid of claim 11 wherein said nucleic acid encodes Apo-2 ligand comprising amino acid residues 41-281 of Figure 1A.
14. The nucleic acid of claim 11 wherein said nucleic acid encodes Apo-2 ligand having amino acid residues 1-281 of Figure 1A.
- 30 15. A vector comprising the nucleic acid of claim 11.
16. A host cell comprising the vector of claim 15.
17. A method of producing Apo-2 ligand comprising culturing the host cell of claim 16 and recovering the Apo-2 ligand from the host cell culture.
- 35 18. A composition comprising Apo-2 ligand and a pharmaceutically-acceptable carrier.
19. The composition of claim 18 wherein said Apo-2 ligand comprises amino acid residues 114-281 of Figure 1A.
20. A composition useful for stimulating mammalian cell apoptosis comprising an effective amount of Apo-2 ligand in a pharmaceutically-acceptable carrier.

21. A method of inducing apoptosis in mammalian cells comprising exposing mammalian cells to an effective amount of Apo-2 ligand.

22. A method of treating a mammal having cancer, comprising administering to a mammal diagnosed as having cancer an effective amount of Apo-2 ligand.

5 23. The method of claim 22 wherein said cancer is breast cancer or colon cancer.

24. An article of manufacture, comprising:

a container;

a label on said container; and

a composition contained within said container;

10 wherein the composition includes an active agent effective for inducing apoptosis, the label on said container indicates that the composition can be used to induce apoptosis, and the active agent in said composition comprises Apo-2 ligand.

25. The article of manufacture of claim 24 further comprising instructions for administering the Apo-2 ligand to a mammal.

15 26. An antibody which binds to Apo-2 ligand.

27. The antibody of claim 26 wherein said antibody is a monoclonal antibody.

28. The antibody of Claim 27 having the biological characteristics of the 1D1 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_.

20 29. The antibody of Claim 27 wherein the antibody binds to the same epitope as the epitope to which the 1D1 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_ binds.

25 30. The antibody of Claim 27 having the biological characteristics of the 2G6 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_.

31. The antibody of Claim 27 wherein the antibody binds to the same epitope as the epitope to which the 2G6 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_ binds.

30 32. The antibody of Claim 27 having the biological characteristics of the 2E11 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_.

33. The antibody of Claim 27 wherein the antibody binds to the same epitope as the epitope to which the 2E11 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_ binds.

35 34. The antibody of Claim 27 having the biological characteristics of the 5C2 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_\_.

35. The antibody of Claim 27 wherein the antibody binds to the same epitope as the epitope to which the 5C2 monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC \_\_\_\_ binds.

36. A hybridoma cell line which produces the antibody of Claim 27.

37. The hybridoma of Claim 36 comprising American Type Culture Collection Accession Number ATCC \_\_\_\_.

38. The hybridoma of Claim 36 comprising American Type Culture Collection Accession Number ATCC \_\_\_\_.

39. The hybridoma of Claim 36 comprising American Type Culture Collection Accession Number ATCC \_\_\_\_.

40. The hybridoma of Claim 36 comprising American Type Culture Collection Accession Number ATCC \_\_\_\_.

41. A non-human, transgenic animal which contains cells that express nucleic acid encoding Apo-2 ligand.

42. The animal of claim 41 which is a mouse or rat.

43. A non-human, knockout animal which contains cells having an altered gene encoding Apo-2 ligand.

44. The animal of claim 43 which is a mouse or rat.





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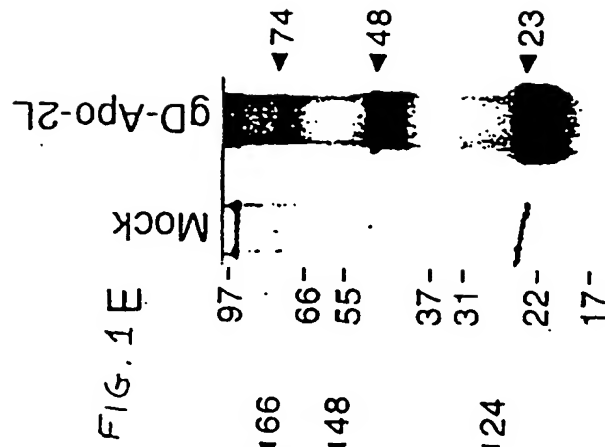


FIG. 1E

FIG. 1D

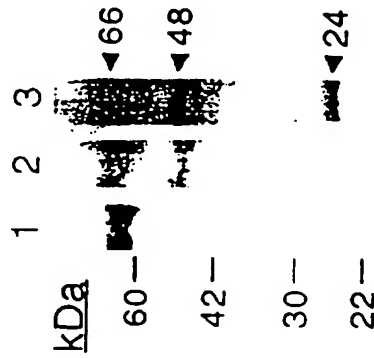


FIG. 1C

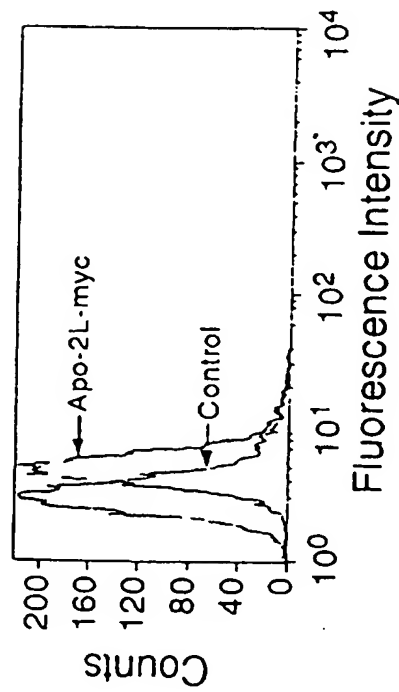


FIG. 2 A 9D cells

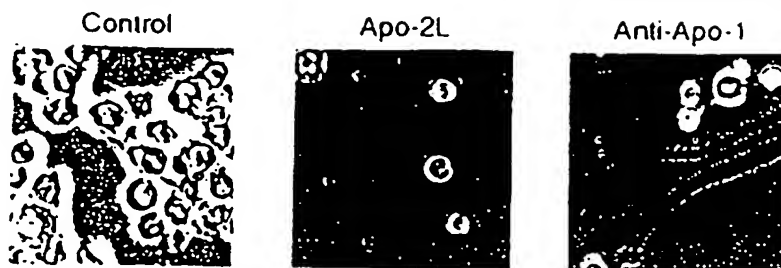


FIG. 2 B 9D cells

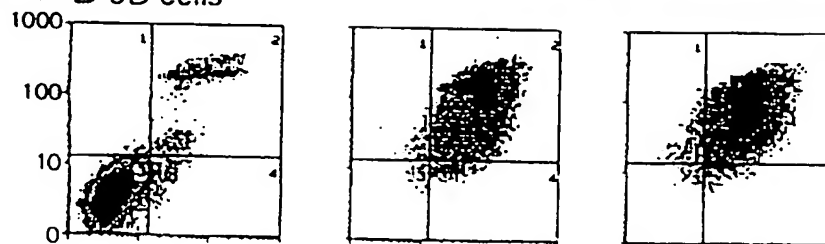


FIG. 2 C Raji cells

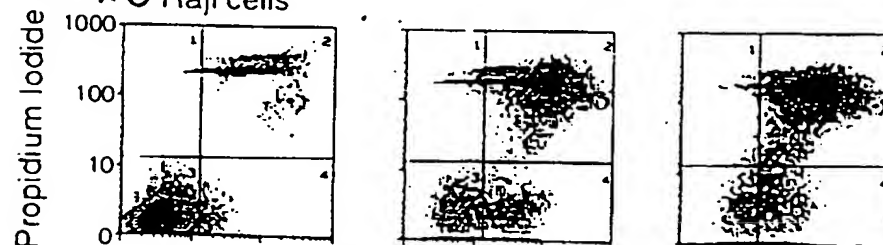


FIG. 2 D Jurkat cells

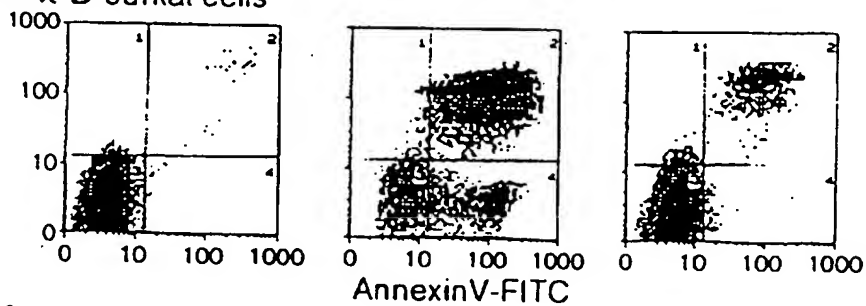
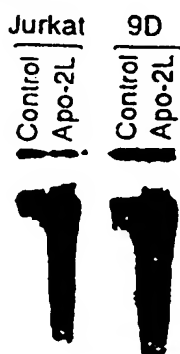


FIG. 2 E



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FIG. 3 A

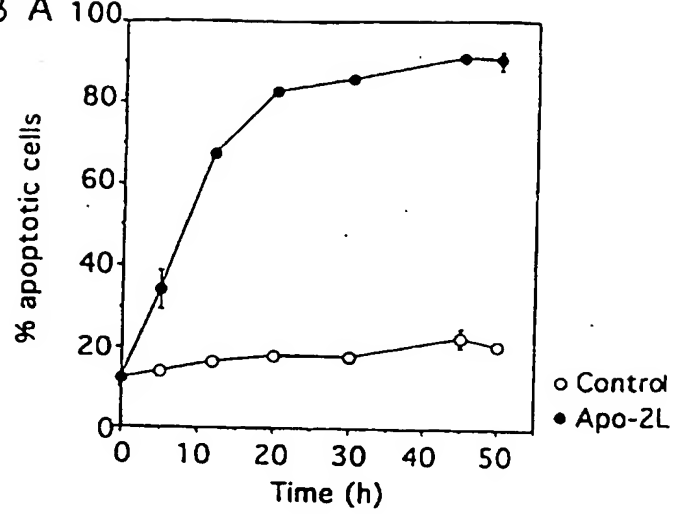


FIG. 3 B

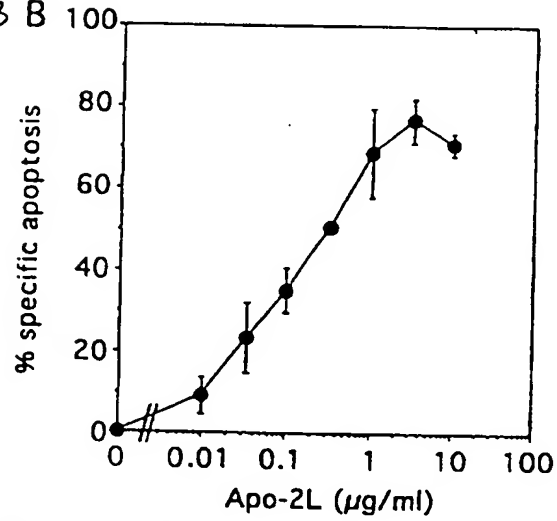
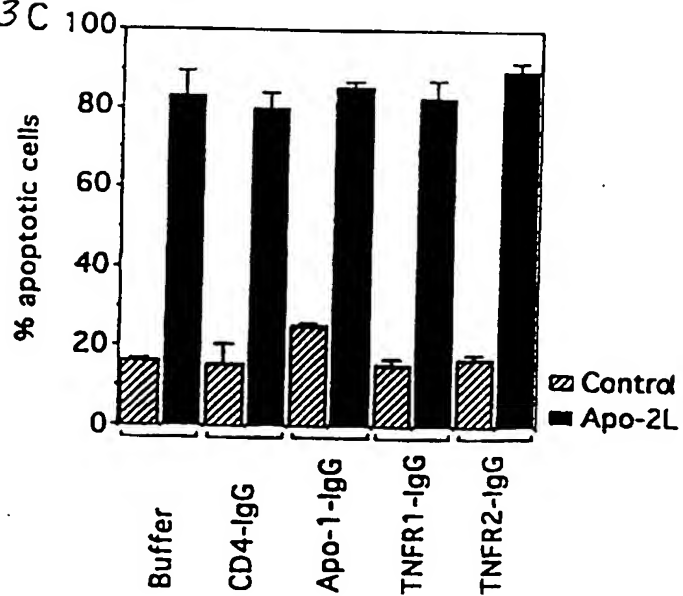


FIG. 3 C



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FIG. 4

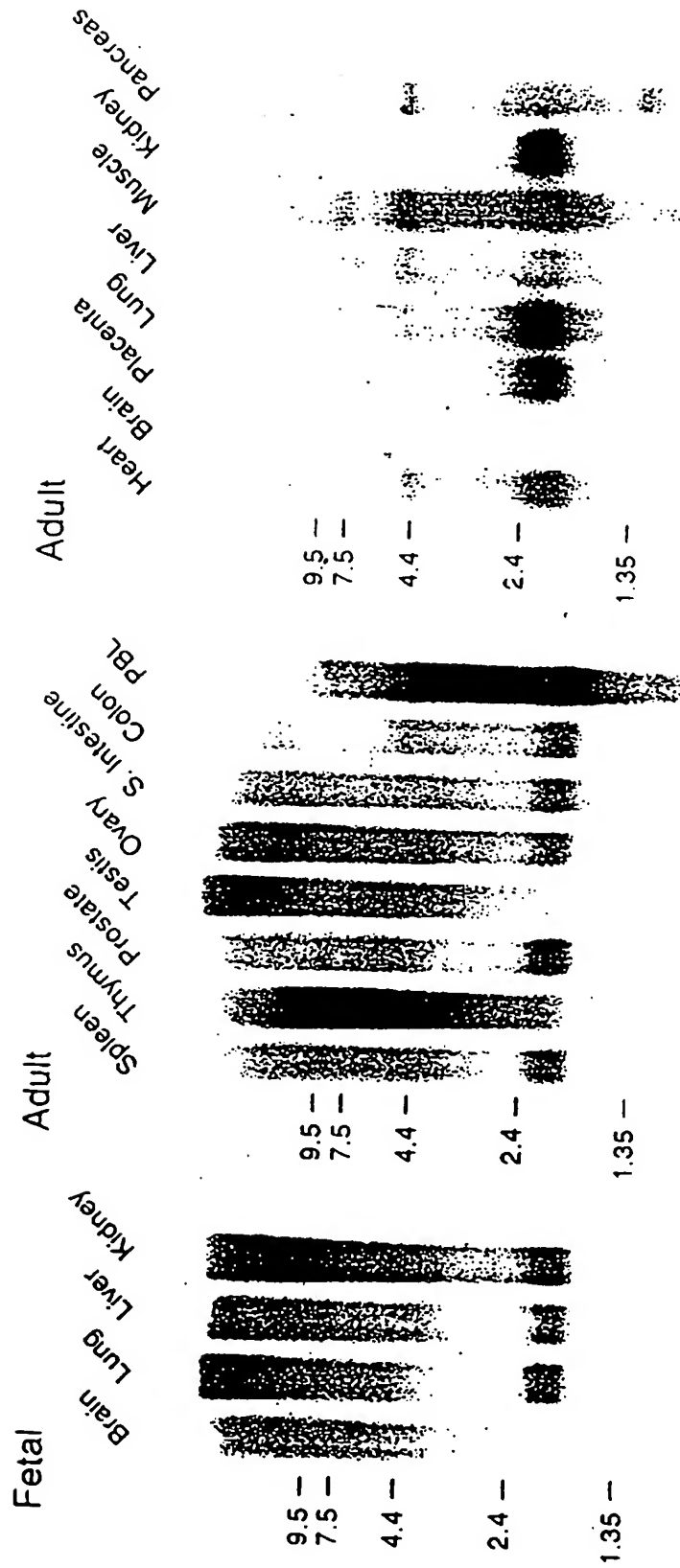
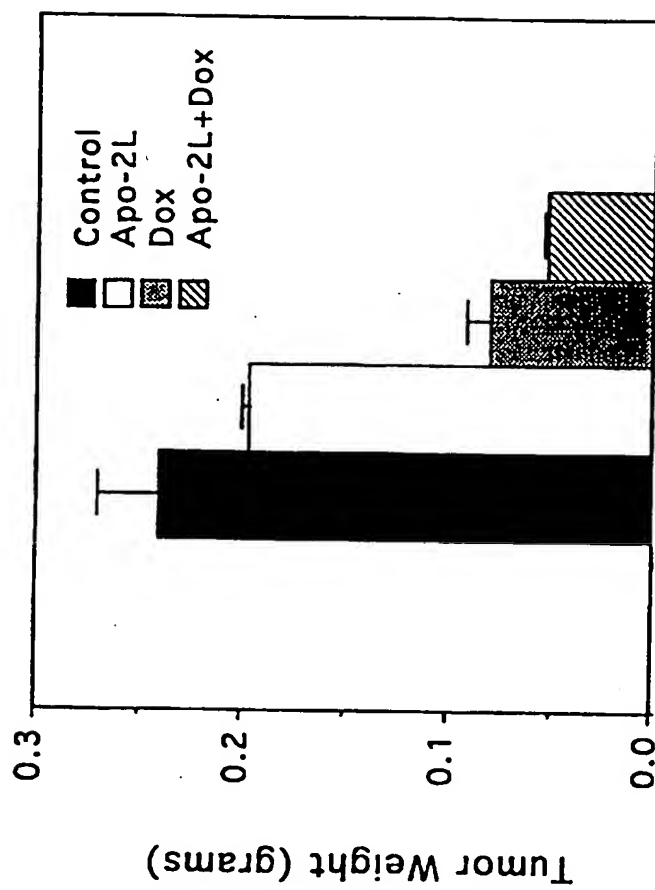


Fig. 5



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Fig. 6

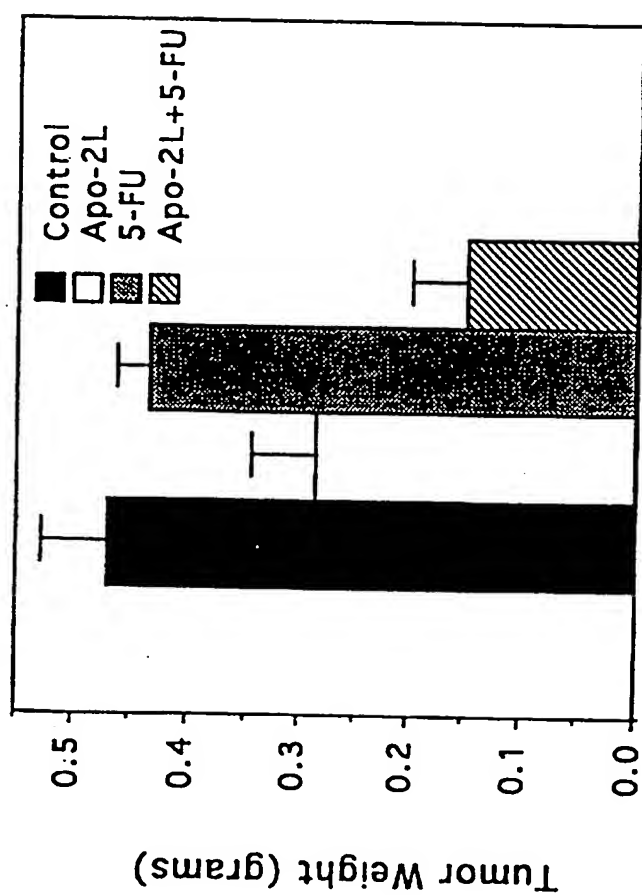
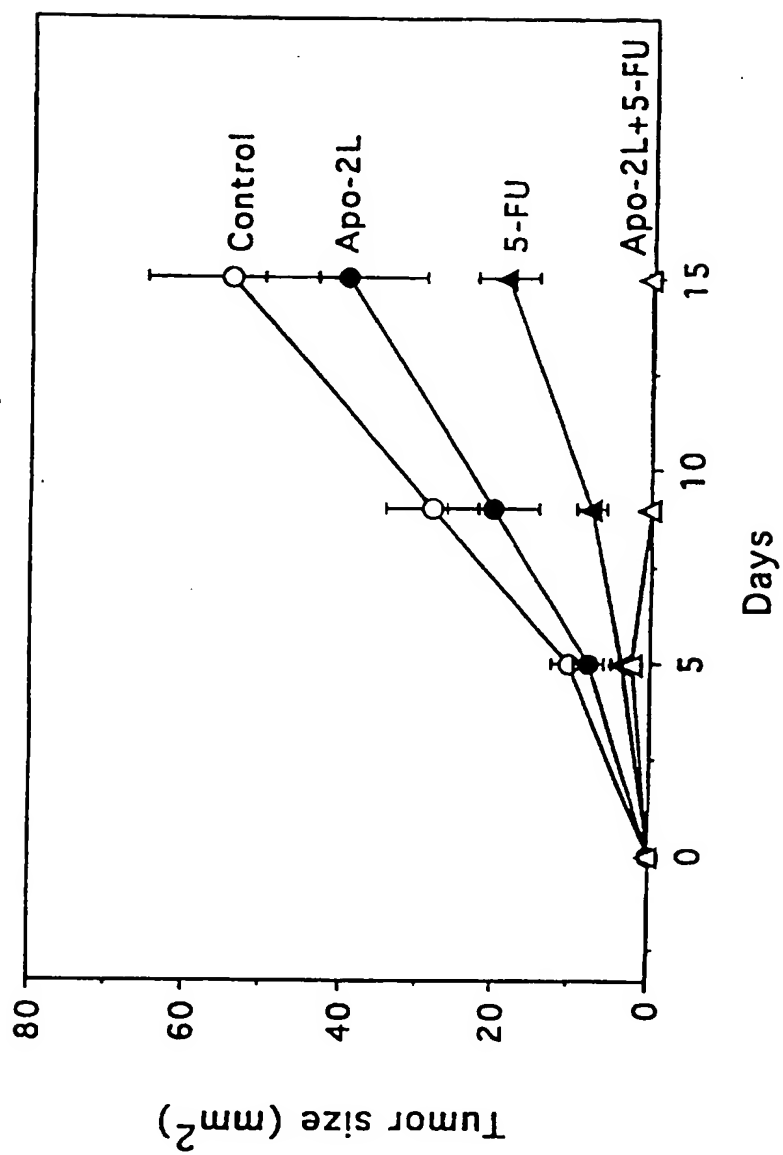
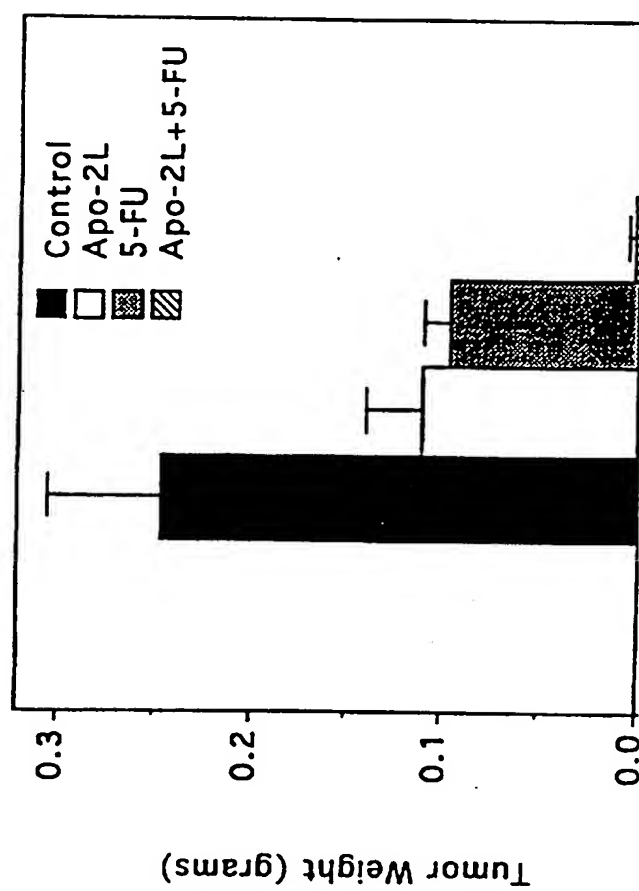


Fig. 7



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Fig. 8





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Fig. 9

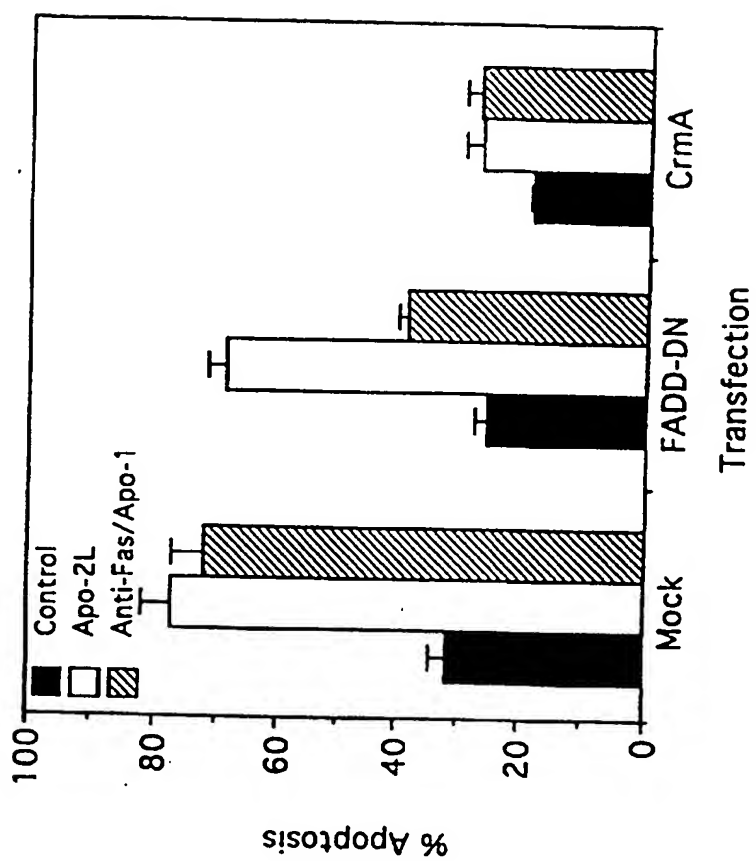
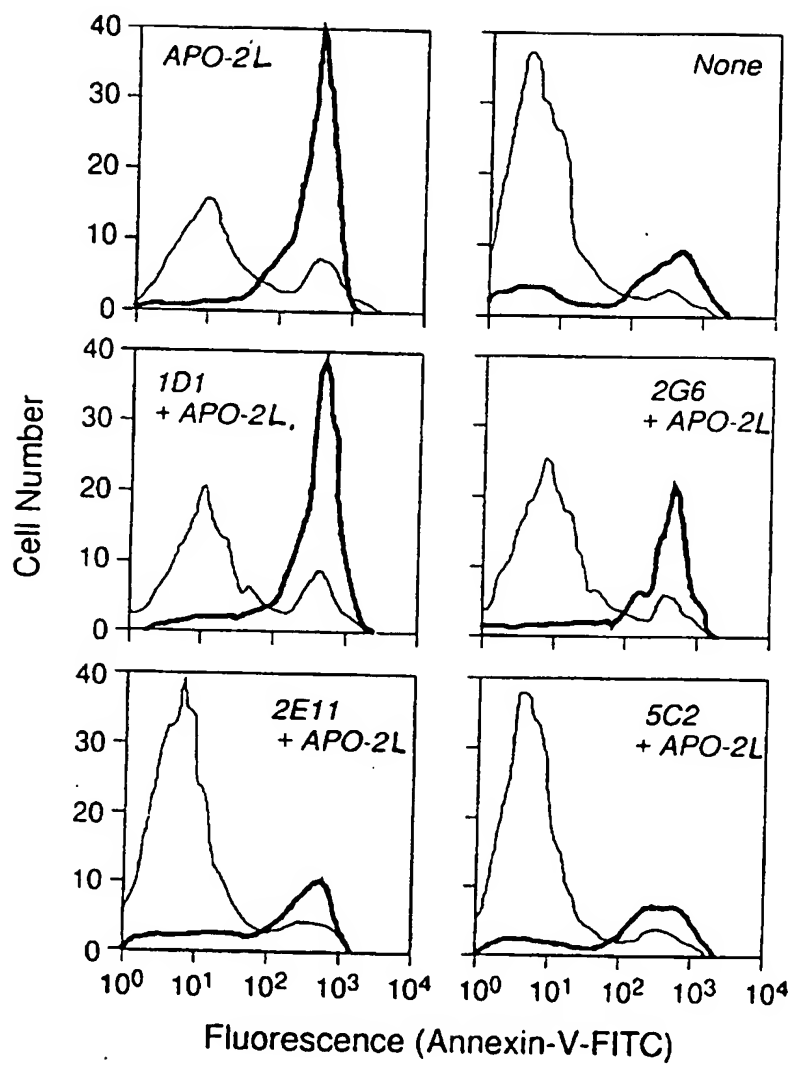


Fig. 10



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Fig. 11

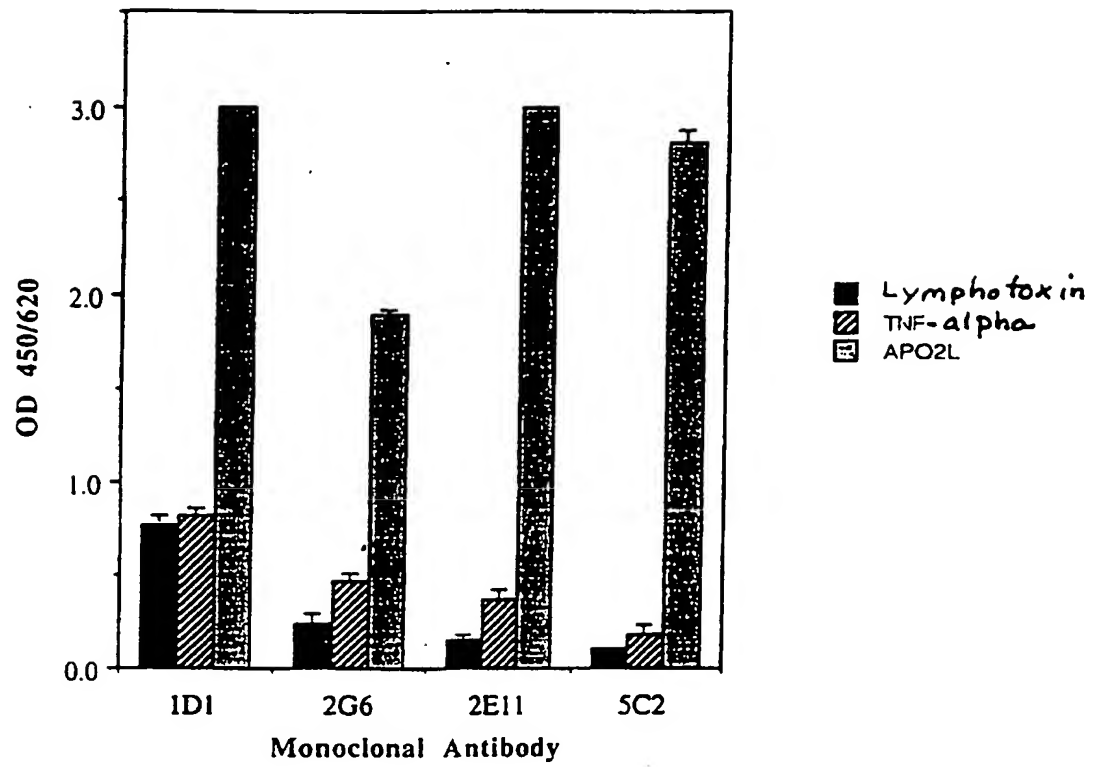
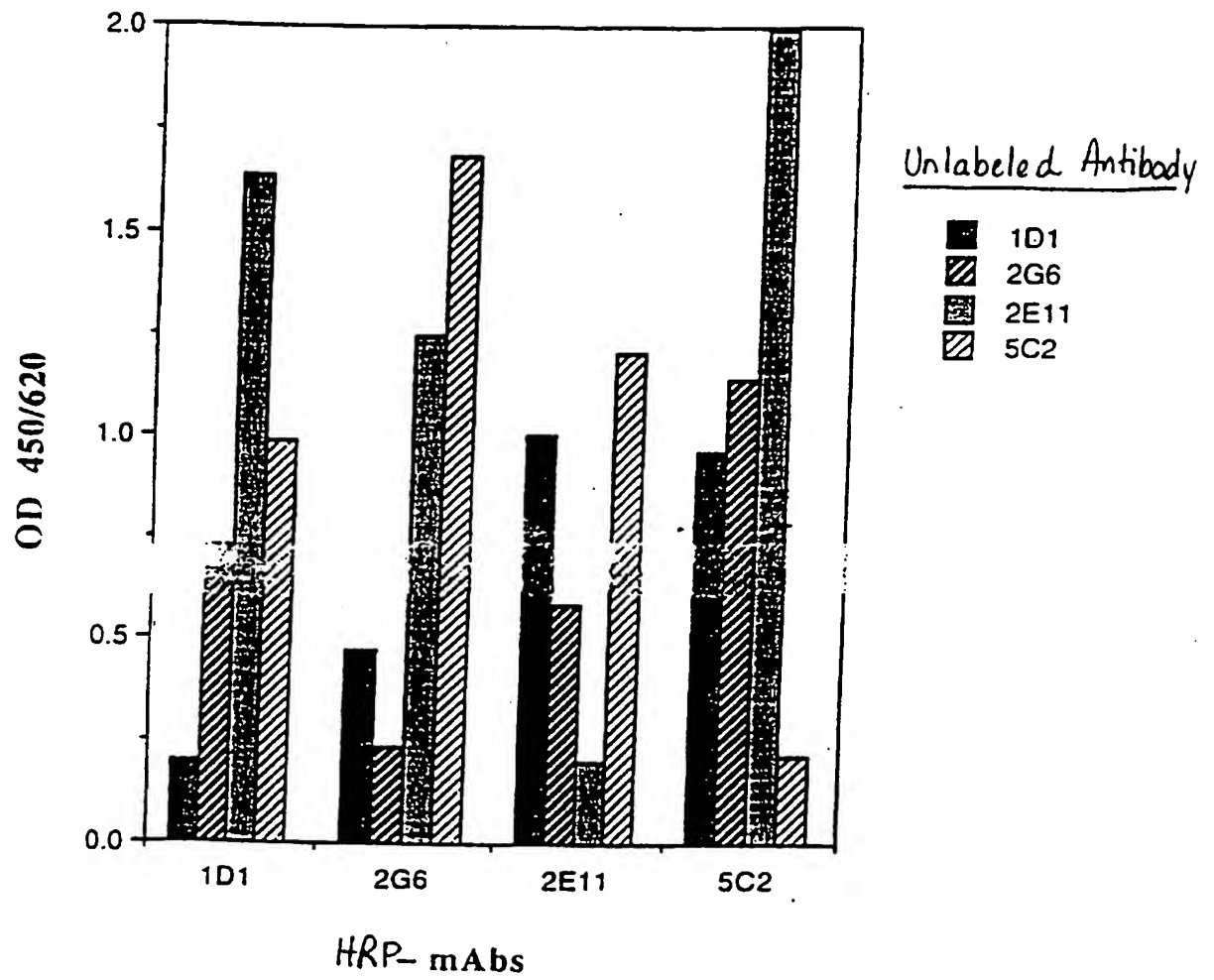
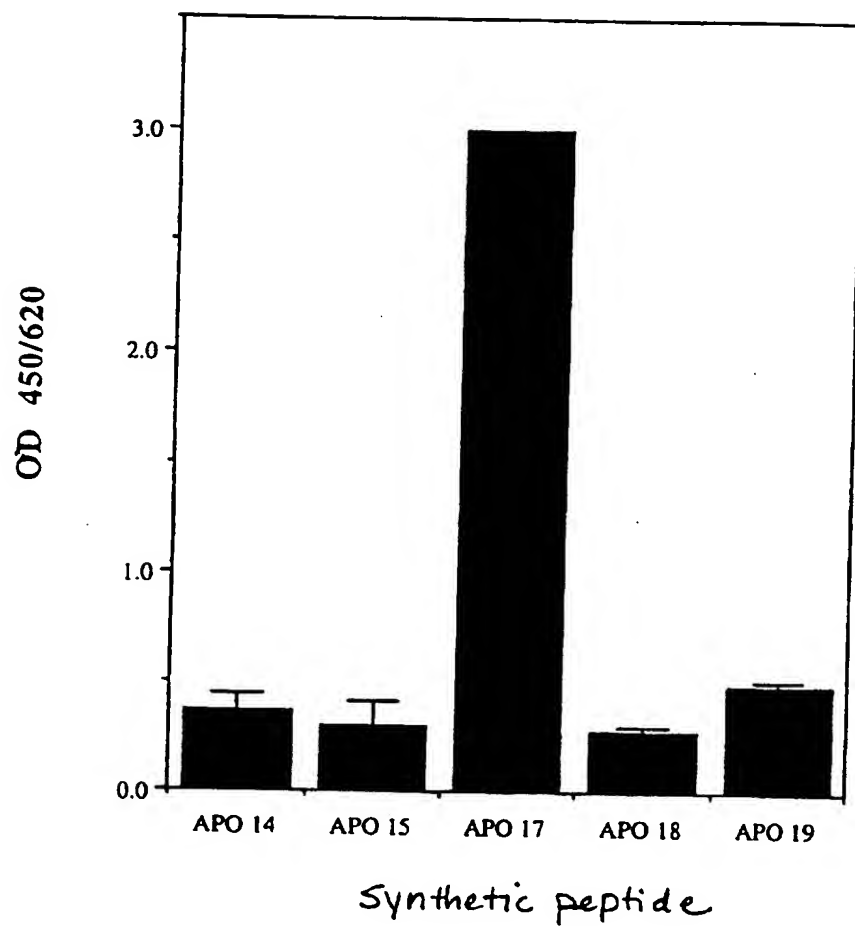


Fig. 12



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Fig. 13



# INTERNATIONAL SEARCH REPORT

International Application No

PC: /US 97/00272

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/28 C07K14/525 C12N15/62 C12N15/86 C12N5/10  
A61K38/19 C07K16/24 C12N5/18 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>IMMUNITY, vol. 3, no. 6, December 1995, pages 673-682, XP000672297 STEVEN R. WILEY ET AL.: "Identification and characterization of a new member of the TNF family that induces apoptosis" see abstract see page 673, right-hand column, paragraph 4 - page 674, left-hand column, paragraph 2; figure 1 see page 674, left-hand column, last paragraph - page 675, left-hand column, paragraph 1 see page 676, right-hand column, paragraph 4 - page 678, left-hand column, paragraph 2</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-44

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
23 May 1997	03.06.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  Montero Lopez, B

# INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 97/00272

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 22, 31 May 1996, MD US, pages 12687-12690, XP002031265 ROBERT M. PITTI ET AL.: "Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family" see abstract see page 12688, left-hand column, paragraph 1 - page 12690, right-hand column, paragraph 1 ---	1-44
P,X	EUROPEAN CYTOKINE NETWORK, vol. 7, no. 2, April 1996 - June 1996, page 159 XP000672223 A. ASHKENAZI ET AL.: "Induction of apoptosis by APO-2 ligand, a new member of the tumor necrosis factor cytokine family" see the whole document. ---	1,21
E	WO 97 01633 A (IMMUNEX CORPORATION) 16 January 1997 see page 2, line 24 - page 3, line 16 see page 4, line 13 - page 5, line 6 see page 8, line 5 - line 15 see page 10, line 7 - page 11, line 6 see page 14, line 13 - page 28, line 34; examples 1,4,5,8,9 -----	1-44

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/00272

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 21, 22, 23  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 21, as far as concerning an in vivo method, and claims 22 and 23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



